Fruits and Vegetables, Detoxification Genes and Intermediate Endpoints in Colorectal Cancer Prevention

Mariken J. Tijhuis

Promotor:
Prof. Dr. Ir. F.J. Kok, hoogleraar Voeding en Gezondheid, Wageningen Universiteit

Co-promotores:
Dr. Ir. E. Kampman, universitair hoofddocent, Sectie Humane Voeding, Wageningen Universiteit
Dr. Ir. J.M.M.J.G. Aarts, universitair docent, Sectie Toxicologie, Wageningen Universiteit

Promotiecommissie:
Dr. J.W. Lampe, Fred Hutchinson Cancer Research Center, Seattle, USA
Dr. H.B. Bueno de Mesquita, Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven
Prof. Dr. A. Bast, Universiteit Maastricht
Prof. Dr. Ir. M.A.J.S. van Boekel, Wageningen Universiteit

Dit onderzoek is uitgevoerd binnen de onderzoeksschool VLAG
(Voeding, Levensmiddelentechnologie, Agrobiotechnologie en Gezondheid)

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. Dr. M.J. Kropff
in het openbaar te verdedigen
op donderdag 10 januari 2008
des ochtends te elf uur in de Aula

Mariken J. Tijhuis

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Thesis Wageningen University – with references – with summary in Dutch

ISBN: 978-90-8504-749-0

Abstract

Background: Fruits and vegetables may counteract the development of colorectal cancer by stimulating the production of detoxification enzymes. Interindividual genetic variation in detoxification enzymes, especially in regulatory regions responsive to fruit and vegetable components, may influence this effect. Two types of detoxification enzymes, that are known to be inducible and polymorphic, are the glutathione S-transferases (GSTs) and NAD(P)H:quinone oxidoreductase (NQO1).

Aim and methods: The aim of the present research was to evaluate fruit and vegetable intake and polymorphisms in the *GSTA1*, *GSTP1* and *NQO1* genes in relation to 1) the expression and enzymatic activity (enzyme phenotype) of GSTA1, GSTP1 and NQO1 and 2) the occurrence of colorectal adenomas. Polymorphisms were determined by PCR-RFLP or pyrosequencing. GST and NQO1 enzyme phenotypes were measured in rectal biopsies and white blood cells among 90 endoscopy-based subjects and their fruit and vegetable intake was assessed by food record. Colorectal adenoma risk was estimated from a population of 750 adenoma cases and 700 endoscopy-based controls and their fruit and vegetable consumption was measured by food frequency questionnaire.

Results: Overall, GST or NQO1 activity was not increased by fruit and vegetable intake. Rectal GST isoenzyme levels did appear to differ between those who had and had not consumed certain fruit and vegetable subtypes. GST and NQO1 activity was most strongly influenced by the *GSTP1* 313G>A and *NQO1* 609C>T polymorphisms, respectively. There was no or only low correlation between rectal and white blood cell activities. The combination of the low activity *GSTP1* or low expression *GSTA1* genotypes and higher than median cruciferous vegetable intake resulted in a higher colorectal adenoma risk (OR 1.76, 95%CI 1.16-2.69). Also, the combination of the low activity *NQO1* genotype and smoking resulted in a higher colorectal adenoma risk (OR 1.96, 95%CI 1.40-2.76). In addition, polymorphism combinations of NQO1 and transcription factor NFE2L2 increased adenoma risk.

Conclusions: Fruits and vegetables do not seem to confer protection against colorectal adenomas through the GST and NQO1 detoxification systems. Common genetic variants in regulatory regions of *GSTA1*, *GSTP1* or *NQO1*, responsive to fruits and vegetables, may not exist. In combination with dietary, other lifestyle or other genetic factors, common genetic variants in the GSTA1, GSTP1 or NQO1 detoxification genes, resulting in lower enzyme expression or activity, may be associated with higher adenoma risk. Currently, however, this does not justify the tailoring of fruit and vegetable advice based on genotypes.

Contents

PROBLEM DEFINITION

Colorectal cancer is one of the most common cancers in the Netherlands, accounting for 13-14% of new cancer cases (i.e ~10.000 individuals) in 2003, affecting men and women almost equally[1]. Five-year survival is ~50%, varying greatly depending on tumor stage[1]. Incidence rates vary ~25-fold around the world, with the highest rates in the developed world[2]. Cancer is a disease originating in the DNA. Most colorectal cancer-causing mutations are somatic[3]. Unfavourable lifestyle factors create the environment in which these mutations have a higher chance of occurring and colorectal cancer is thus potentially avoidable[4, 5]. One protective factor, of inconsistent magnitude, is a high consumption of fruits and vegetables[6, 7]. One proposed mechanism is that fruit and vegetable constituents stimulate the production of beneficial detoxification enzymes[8]. These enzymes act on carcinogens and facilitate their detoxification and excretion out of the body. As a consequence, DNA damage leading to tumor formation is prevented. However, these enzymes are subject to genetic variation affecting their levels or activity and possibly also causing individuals to respond differently to fruits and vegetables. This hereditary component of low-penetrant disease prevention may obscure epidemiological associations between fruit and vegetable consumption and cancer.

This thesis describes the results of two human observational studies investigating colorectal adenomas and rectal enzyme levels and activities (enzyme phenotype) in relation to fruit and vegetable consumption and genetic variation (genotype) in two types of biotransformation enzymes: GSTs and NQO1. The present chapter describes, narrowing in on potential mechanisms and using mostly results from studies in humans, the relevant existing knowledge, its gaps and the rationale for the present studies. It ends with the research questions and the thesis outline.

Chapter 1

Introduction

FRUITS, VEGETABLES AND COLORECTAL TUMORS

Epidemiology. High consumption of **fruits and vegetables** has been reported to protect against **colorectal cancer**[6, 9]. Special attention as possible cancer-protective components of the human diet has been given to citrus fruits, green leafy vegetables, allium vegetables and especially cruciferous vegetables[10, 11]. A convincing protective effect is supported by most case-control studies, but less so by more recent prospective cohort studies[6, 7, 12, 13], conferring uncertainty to the degree of risk reduction by consumption of fruits and vegetables. Design-related reasons for the inconsistencies include recall bias or actual change in dietary habits due to disease state in case-control studies[14]. Time-related issues may be found in content of protective components due to use of fertilizer, pesticides and continued genetic selection on plant yield[15-17]. More general reasons for the non-significance of a protective effect are random measurement errors in food frequency questionnaires (FFQs)[14, 18] with consequent inability to detect differences, variability of exposure due to differences in plant cultivars, industrial handling or cooking methods[19-21 22] and variability of human metabolism, which may all attenuate a true relative risk and lead to an obscured association.

A number of studies have investigated **colorectal adenomas** as the endpoint in relation to fruit and vegetable consumption. Adenomas are the proposed intermediates in the multistage process of colorectal carcinogenesis[7, 23], interesting from a viewpoint of primary and secondary prevention[24], relatively close in time to relevant lifestyle exposure and relatively abundant[25]. This abundance increases the practical and financial feasibility of adenoma studies, but limits their specificity: an estimated 85% of adenomas do not progress to carcinomas[26]. Adenoma case-control studies are thought to suffer less from recall-bias than cancer case-control studies, because adenomas are relatively asymptomatic. The association between fruit and vegetable consumption and colorectal adenomas has mostly been investigated in case-control design[27-37]. Most studies report inverse associations, which are significant for consumption of total vegetables[29-31, 33] and/or total fruits[28, 31, 32] and fruit juice[34]. Two cohort studies, the Health Professionals' Follow-up Study[36] and the Nurses' Health Study[37], report a decreased adenoma risk with frequent fruit but not vegetable consumption.

Potential mechanisms. The fact that recent epidemiological studies find only weak protective or null associations does not exclude the possibility that specific fruit and vegetable constituents have anticancer properties. A number of fruit and vegetable constituents have been postulated as causative protective factors. Fruits and vegetables are rich in fiber and contain vitamins and a large variety of bioactive secondary metabolites, so-called **phytochemicals**[38-40]. These plant

components help plants to respond to wounding, pathogens and pests, and a changing environment and trigger a range of effects in their consumers. Phytochemicals can be divided into several families: polyphenolics (among which are the flavonoids), terpenes (among which are the carotenoids), sulfides (among which are the isothiocyanates and allylsulfides) and saponins[41]. Some of the bioactive compounds are more universally present, e.g. the carotenoids in yellow to red coloured plants and chlorophyll in green plants. Some are more typical to certain fruit and vegetable botanical subtypes, e.g. allylsulfides to allium vegetables and glucosinolates to cruciferous vegetables which, in themselves biologically relatively inert, can be hydrolysed by the cruciferous plant enzyme myrosinase to cancer-protective active components such as isothiocyanates and indoles[42]. As a surrogate measure for the intake of these specific phytochemicals, fruits and vegetables can be grouped and evaluated botanically[43].

It is likely that the concerted action of the bioactive compounds, and not just a few so-called "magic bullets", are important in human anti-cancer mechanisms[44]. It is not known how many of the proposed mechanisms[38, 39] are actually relevant for human cancer. Anti-carcinogenic phytochemicals are proposed to inhibit various stages of cancer, which can be broadly divided in actions blocking tumor initiation and those suppressing malignant cell transformation[39, 45]. They are thought to affect **carcinogen activation and detoxification**, DNA repair, cell-cycle progression, differentiation and apoptosis, expression and functional activation of oncogenes or tumor-suppressor genes, angiogenesis and metastasis, and hormonal and growth-factor activity[46-48]. The first mechanism, carcinogen activation and detoxification, involves the biotransformation enzyme system, i.e. the handling of foreign compounds (xenobiotics) in the body. It is the first line of defence against cancer-initiating compounds, in which individuals appear to vary substantially. Narrowing in on this biochemical mechanism and the genes involved, in human context and with specific endpoints, may provide us with observations concerning the possible anticancer properties of fruits and vegetables that cannot be obtained when solely studying the association between fruits and vegetables and cancer.

In conclusion: A diet high in fruits and vegetables may be beneficial for human colorectal cancer prevention, though recent epidemiological studies do not convincingly support this. This may be related to the difficulty of a valid and reproducible assessment of fruit and vegetable intake and their possible bioactive constituents. Also, individual (genetic) aspects of xenobiotic metabolism may play a role.

FRUITS, VEGETABLES AND DETOXIFICATION

Biotransformation enzymes. These enzymes render xenobiotics more hydrophilic in order to facilitate their excretion from the body or make them less reactive, as part of an adaptive response to electrophilic carcinogens and oxidative stress[49]. Two types of reactions are usually distinguished: functional group modification by oxidation, reduction or hydrolysis reactions (referred to as phase I, most importantly represented by the cytochrome P450's), which often result in a more reactive product; and conjugation reactions with endogenous ligands such as glutathione, glucuronic acid and sulfate (phase II), which essentially detoxifies the xenobiotic and facilitates excretion. **Phytochemicals** such as isothiocyanates and allyl sulfides are capable of upregulating detoxification gene transcription thus causing higher levels of these detoxification enzymes, a process known as **induction**[40, 50]. In the field of cancer biochemoprevention, compounds are sought that selectively inhibit phase I enzymes and/or that selectively induce phase II enzymes, so-called monofunctional induction, i.e. to specifically increase detoxification.

Gene regulation. Induction, and gene regulation in general, is complex. A gene consists of two functional parts: a DNA sequence that holds the information about the protein that is produced (coding region) and a physically linked upstream region where the general transcription factors assemble to start transcription, referred to as the **promoter**[51]. The transcription initiation site can typically be found at ~25 nucleotide pairs downstream from the so-called TATA box[51]. In some genes, such as heme oxygenase, γ-glutanyl cysteine synthase, NAD(P)H:quinone oxidoreductase (NQO1) and glutathione S-transferase (GST) P1, **phytochemicals** interact with a specific DNA enhancer sequence in the promoter region, the so-called anti-oxidant or **electrophile-responsive element** (ARE/**EpRE**)[52, 53]. Gene expression is controlled through transcription factors, proteins that bind DNA at regulatory regions. In the regulation of EpRE mediated gene expression, transcription factor **NFE2L2** (official human nomenclature for the more commonly known **Nrf2**) has a central role[54-56]. NFE2L2 is anchored in the cytoplasm by a protein named Kelch-like ECH-associated protein 1 (KEAP1)[57]. **Inducers** disrupt the NFE2L2-KEAP1 complex, resulting in higher levels of free NFE2L2 and its translocation to the nucleus where it binds the EpRE as a heterodimeric complex with other transcriptional regulators[39, 57, 58]. The EpRE is responsive to a wide range of structurally diverse **phytochemicals** and other compounds[57], is both anciently conserved and species specific[59, 60] and many of the proteins whose expression is mediated by the EpRE have an endogenous role in cellular redox status[61]. **Regulatory sequences** are found not only in the near

vicinity of the promoter area, but can also be thousands of nucleotide pairs away from the promoter, upstream or downstream, and form loops towards the promoter DNA to allow interaction of the bound transcription factors with the basal transcription complex on the promoter to modulate the transcription level[51]. Many DNA sequences that serve as recognition sites for the binding of specific gene regulatory proteins have been identified in biotransformation genes. Examples are the Sp1 and AP-1 regulatory protein-binding sites[62]. Gene regulatory proteins can act as activators or suppressors. The combination of the promoter and all the regulatory sequences has been referred to as the "gene control region"[51]. Thus, for induction by fruits and vegetables, the EpRE is important, but other regions can be relevant as well. Two interesting biotransformation systems in this respect are the GSTs and NQO1.

In conclusion: Some fruit and vegetable components, so-called phytochemicals, are thought to induce biotransformation enzymes via regulatory DNA sequences such as the electrophile responsive element (EpRE), enabling the body to respond to genotoxic carcinogens and oxidative stress.

GSTs, NQO1 and Induction by Fruits and Vegetables

Two important types of phase II biotransformation enzymes that are inducible by fruit and vegetable components and contain EpRE's are the GSTs and NQO1. Their upregulation could benefit the colorectum because they metabolize relevant environmental carcinogens. They show differences in tissue distribution and considerable interindividual variation. GSTs are expressed at relatively low levels in the colorectal area as compared to other parts of the digestive system[63]. NQO1 is expressed ubiquitously in all the tissues, with relatively high levels in colon[53].

The **GSTs** have been extensively reviewed[64, 65]. GSTs catalyze the conjugation of xenobiotics with glutathione. They are sensitive to many different compounds[65]. The GST superfamily consists of soluble cytoplasmic (often termed "cytosolic" because they end up in the cytosolic fraction upon isolation), mitochondrial and membrane-bound (often termed "microsomal") GSTs. The "cytosolic" superfamily has been divided in 7 classes, arbitrarily based on sequence similarity, which in general share broad overlap in substrate specificity[66] and of which GSTA, GSTM, GSTT and GSTP are the most well-known. GST expression decreases from the upper to the lower digestive tract, where it makes up less than 0.2% of cytosolic proteins[63].

Total GST activity has been found to be increased in plasma and saliva after cruciferous vegetable consumption[67-69]; It was increased in colon tissue and

peripheral mononuclear cells after a single dose of the synthetic dithiolethione Oltipraz[70], although not after chronic dosing or after broccoli supplements[71]; and an observational study noted a positive association with cruciferous vegetables and citrus fruits in colon tissue[72]. Intervention with green tea catechins (a group of flavonoids) resulted in a higher lymphocyte total GST activity among individuals with lower baseline GST activity, yet in lower lymphocyte total GST activity among those with higher baseline GST activity[73].

GSTA is the most abundant GST in the liver[74]. Within the digestive tract, it is highly expressed in duodenum and small intestine, but lowly in stomach and colon[75]. Among others, GSTA is capable of metabolizing polycyclic aromatic hydrocarbon (PAH) diolepoxides, found in cigarette smoke and grilled and smoked meats[76, 77] and it is the most sensitive GST in metabolizing the carcinogenic heterocyclic amine (HCA) N-acetoxy-PhIP, the most mass abundant HCA in meats cooked at high temperature[76, 78]. GSTA has been shown to inhibit the binding of N-acetoxy-PhIP to DNA efficiently[76, 79].

GSTA levels have been noted to be inducible by cruciferous vegetables in human intervention studies in rectum[80]. Upregulation was also seen in plasma, which is thought to reflect normal hepatic expression through normal hepatocyte turnover[81], among males[82, 83] and a genetic subgroup in females[84]. These notions are supported by studies in different human (cancer) cell lines reporting increased transcription with the isothiocyanate sulphoraphane: HT29 and Caco-2 (colonic adenocarcinoma), HepG2 (hepatoma), and several prostate cancer lines[85-87]. In human hepatocytes, 1,2-dithiole-3-thione and its 5-(2-pyrazinyl)-4-methyl derivative, oltipraz increased GSTA transcription (but not GSTP1 transcription)[88]. In an observational study of the upper gastrointestinal tract, high intake of vegetables was associated with higher duodenal GSTA[89]. However, an EpRE has not been identified in the regulatory region of the genes encoding human GSTA[90] and the nucleotide elements responsible for induction are not known. In contrast, an intervention study with green tea catechins noted a lower plasma GSTA level among those with higher baseline levels[73].

GSTP1 is the most abundant GST in the colorectum[80]. It metabolizes, among others, PAH diolepoxides, HCAs and nitroso-compounds[91]. It has been shown to moderately inhibit the binding of N-acetoxy-PhIP to DNA[76, 79] and to inhibit its enzymatic activation in a human prostate adenocarcinoma cell line[92]. GSTP1 is overexpressed in the majority of human tumors, among which colon tumors[64, 93].

Intervention studies have noted upregulation of GSTP1 with Brassica vegetables in rectum[80] and in plasma[68, 69] and with green tea catechins in lymphocytes among individuals with lowest baseline level tertile[73], yet found

downregulation in lymphocytes after mixed vegetables[94]. In an observational study, downregulation in rectum with habitual Allium consumption was seen[72]. In another observational study, high intake of vegetables was associated with higher GSTP1[89].

GSTM1 and **GSTT1** have gained the most research attention so far, not so much from the perspective of induction by fruit and vegetable components, but from the perspective of the clear individual variation in their expression, which will be explained under the next heading. **GSTT1** is highly expressed in the colorectum[95] whereas **GSTM1** is not[80]. In this thesis, the main focus of attention is on GSTA1 and GSTP1.

NQO1. Quinones are aromatic compounds widely present endogenously and in our environment: as components of plants and exhaust fuels or used in dyes, photography and chemotherapy[96, 97]. If reduced by one electron, the reactive intermediate might give rise to the formation of reactive oxygen species, and DNA and protein adducts[98]. Thus it is thought that in evolution there has been strong selection for the development of enzymes that catalyze the two-electron reduction of quinones. NQO1 hydrolyses quinones to hydroquinones[99]. There is debate whether NQO1 should be termed a phase I or phase II biotransformation enzyme[100]. Arguments in favour of phase II are that NQO1 does not introduce new functional groups, it is often induced co-ordinately with other phase II enzymes and it exerts protective functions[101]. However, besides its detoxifying function it can also bioactivate[102]. Confusingly, in the (older) literature, NQO1 has also been referred to as "DT-diaphorase"[97]. The use of 'diaphorase' to denote a 'coenzyme' was widespread in earlier enzymatic studies and 'DT' refers to *D*PNH (NADH) and *T*PNH (NADPH) because the enzyme works well with both.

In human intervention studies, NQO1 activity increased in saliva after broccoli consumption[67]. *NQO1* mRNA increased in peripheral mononuclear cells and colonic biopsies after single administration of the synthetic dithiolethione Oltipraz[70], but not after chronic dosing[103]. In cell studies, indoles and isothiocyanates increased NQO1 protein in colon cancer cells LS-174[104]; Synthetic dithiolethione 1,2-dithiole-3-thione (D3T) increased NQO1 activity in mitogenized human lymphocytes[101] and several human tumor cell lines[105]; Garlic organosulfur compounds enhanced NQO1 gene expression in HepG2 cells[106]; β-Carotenes (retinol and retinoic acid but not lycopene), α-tocopherol and ascorbic acid induced NQO1 (but not GST) activity in human colon cancer cells, colo205[107]; And several compounds, among which isothiocyanates, catechol, epigallocatechin gallate (EGCG), quercetin and curcumin, induced NQO1 in a human prostate cancer cell line, LNCaP; and human hepatoblastoma cell line, HepG2[86, 108].

In conclusion: The biotransformation enzymes GSTP1, GSTA1 and NQO1 are interesting in light of colorectal cancer risk modification by fruit and vegetable consumption because they are known to be under transcriptional control of an EpRE (NQO1, GSTP1) or are otherwise inducible (GSTA1) by plant components, such as isothiocyanates, flavonoids, carotenoids, sulfides and phenols. The inducibility of human GST levels by consumption of fruits and vegetables, such as cruciferous vegetables, has been shown in vivo. However, results are inconsistent or only one GST isoenzyme or fruit and vegetable subtype was studied, most often in blood. For NQO1 there are little in vivo data in humans.

GENETIC POLYMORPHISMS IN GSTS AND NQO1

The first draft of the human DNA sequence was published in 200[109-111], the greatly anticipated result of the **Human Genome Project**[112]. The HGP is the largest and most ambitious research project in the history of biology, started officially in 1988, with the aim to obtain a detailed map and complete DNA sequence of the human genome. The working draft of the euchromatic sequence was reported in 2001[110, 113] and in 2004 a major improvent of this working draft was published[111]. The gene count, once estimated to be 100.000, was corrected to 20-25.000. Though human DNA is the same between individuals for > 99.9% of the 3 billion base pairs, interindividual sequence variation exists throughout the genome, such as deletions, copy number variants, variable tandem repeats and, most common, single nucleotide differences called **single nucleotide polymorphisms** (**SNPs**). Another large project, the HAPMAP project[114], has been devoted to compare the genetic sequences of different individuals to identify chromosomal regions where genetic variants are shared. SNPs have been estimated to occur on 10 million locations[114], but this can be reduced to approximately 250.000 haplotype bins in Caucasians[115, 116]. Most SNPs are non-functional on a molecular level, i.e. a nucleotide difference does not lead to an amino acid change or occurs in a sequence without regulatory consequences. In some cases, they are functional on a molecular level, i.e. the level or activity of a gene product is changed, with possible consequences for susceptibility to disease. Unlike rare hereditary mutations in key genes such as APC or mismatch repair genes, with a penetrance of 80 – 100%[3], genetic differences in detoxification genes do not so much affect the risk of cancer per se, but are associated with a modified risk in relation to exposure to a potential carcinogen or cancer chemoprotectant[117].

***GSTA1* polymorphisms.** The *GSTA* genes are clustered on chromosome 6p12 and consist of 5 genes: *GSTA1 – A5* and 7 pseudogenes[90]. The GSTA protein consists of homo- and heterodimers of the proteins encoded by the *GSTA1* and *GSTA2* genes. The GSTA1 subunit is the most prevalent subunit in liver and most

other tissues[74, 81], but in the colon they are approximately equally low[75]. A lot less is known about *GSTA3* and *GSTA4* and the *GSTA5* gene may not be expressed at all[90]. *GSTA1* consists of 7 exons, spanning ~12kb and encoding a total of 222 amino acids. A number of SNPs have been reported in the *GSTA1* gene[118-120], the most important to date being the haplotype consisting of 3 linked base substitutions in the proximal promoter: -567T>G, -69C>T, and -52G>A resulting in a ~4-fold lower expression in human liver samples (but not in pancreas)[119]. It has been referred to as *GSTA1**A and *GSTA1**B, has mostly been genotyped at position -69 and has an allele frequency of ~40% in Caucasians[90]. Its functionality has been shown in luciferase reporter constructs and has been attributed to the -52G>A SNP causing alteration of the binding of transcription factor Sp1[119, 121]. Compared to homozygous wild-type and heterozygous individuals, individuals carrying two variant *GSTA1* alleles were shown to have higher lymphocyte PhIP-adduct levels in a subgroup of young individuals[122] and those consuming well-done meat frequently (>2 servings/wk) were observed to have higher colorectal cancer risk[123].

***GSTP1* polymorphisms**. The *GSTP1* gene is located on chromosome 11q13. It is a single gene, consisting of 7 exons, spanning ~2.8 kb and encoding a total of 209 amino acids. Functional coding polymorphisms in the *GSTP1* gene have been reported: an A>G SNP at position 313 (termed *GSTP1**B) and an additional C>T SNP at position 341 (containing both SNPs and termed *GSTP1**C)[124]. Both nucleotide changes translate into amino acid changes (isoleucine to valine and alanine to valine, respectively) and both amino acid changes are in the electrophilic substrate-binding site of the GSTP1 protein, reducing its catalytic activity[124, 125]. The GSTP1*B allele affected GSTP1 activity in lung tissue[126] and total GST activity (CDNB) in erythrocytes[127]. The *GSTP1* *B and *C allele frequencies are ~26% and 7% in Caucasians[128]. The variant *GSTP1* 313 G-allele was associated with higher DNA damage in breast tissue (especially in combination with the A-463 variant in the myeloperoxidase gene; but not *GSTT1*, *GSTM1* or *NQO1* variants)[129], and in mononuclear white blood cells in smokers with the *GSTM1*- genotype[130].

In the regulatory region of *GSTP1*, a functional haplotype has recently been identified which displays higher basal *GSTP1* mRNA and protein expression. In this Caucasian study population of 40 individuals, there were three heterozygotes and one homozygote[131].

***GSTM1* and *GSTT1* polymorphisms.** *GSTM1* and *GSTT1* are located on chromosomes 1p13.3 and 22q11.2, respectively. In about 50% and 20% of Caucasians, respectively, the genes are partially deleted on both alleles, resulting in absence of activity[128, 132]. The switch from a vegetarian diet to a high-meat diet significantly increased the amount of DNA single-strand breaks in exfoliated

colonic mucosa cells among individuals with a *GSTM1-* genotype but not among those with *GSTM1+* genotype[133]. A Human Genome Epidemiology (HUGE) review did not observe consistent associations between *GSTM1* or *GSTT1* genotype and colorectal cancer[132].

Only in recent years laboratory assays discerning individuals with one and two alleles present have become feasible and more common practice[134-136]. Allele frequencies of 0.77 for *GSTM1-*[135] and 0.43 for *GSTT1-*[134] in Caucasians have been reported. The resulting study data indicate that the former grouping together of heterozygotes and homozygous wild-types does not do justice to the phenotypic differences that appear to exist. In erythrocytes, *GSTT1* activity was found to correlate with number of alleles, with an intermediate activity (towards dichloromethane) for the *GSTT1* heterozygous genotype[134]. Having one or two inactive *GSTT1* alleles was associated with 40% increased left-sided advanced colorectal adenoma risk, among smokers[136]. For left-sided advanced colorectal adenomas, risks were essentially the same for *GSTM1* heterozygotes and homozygous null variants, both were decreased by 40%[136]; In breast cancer risk, a similar observation was made[135].

***NQO1* polymorphisms.** The *NQO1* gene is located on chromosome 16q22.1. It contains 6 exons, spanning ~20 kb, totalling 274 amino acids. There are two known functional polymorphisms; one C>T substitution at cDNA position 609, with an allele frequency of ~20% in Caucasians[137], resulting in rapid degradation of the variant protein[138] and loss of enzymatic activity as shown in saliva[139], lung tissue[140] and bladder tumors[141]; and one C>T substitution at position 465, with an allele frequency of ~5% in Caucasians, resulting in an alternatively spliced transcript lacking exon 4 which contains the quinone binding site[142, 143]. The *NQO1* 609C>T SNP appears to be associated with an increased cancer risk in benzene-exposed populations[144], but the association is less clear for other populations[145, 146].

In conclusion: Genetic variation exists in GST and NQO1 detoxification enzymes. There is little evidence for an important role in cancer risk for genetic variation in itself. It is thought to be relevant in combination with exposure to specific pro- or anti-carcinogens. The specific impact of GST and NQO1 polymorphisms on colorectal phenotypes, e.g. enzymatic capacity or tumour formation, is not sufficiently known. Little is known about functional polymorphisms in GST or NQO1 regulatory regions, which could be particularly important because they may lead to differences in (constitutive and inducible) gene expression and therefore affect susceptibility to environmental exposure and cancer-chemoprotective efficacy.

FRUIT AND VEGETABLE CONSUMPTION AND POLYMORPHISMS IN GSTS AND NQO1: INTERACTIONS WITH RESPECT TO HUMAN COLORECTAL CANCER-RELATED ENDPOINTS

Polymorphisms are known to occur in **regulatory** regions, as has been described for *GSTA1*, *GSTP1* and other genes[119, 131, 147, 148]. It is conceivable that regulatory sequence variation exists specifically in nucleotide sequences responsive to phytochemicals, for example in the EpRE[149], causing differences in the effectiveness of these cancer-preventive constituents among individuals. This may be true in a suppressive manner for the recently described *GSTP1* haplotype with increased constitutive expression[131]. There, strikingly, the presumed inducers sulforaphane, EGCG and benzyl isothiocyanate (BITC) *in vitro* decreased the haplotype promoter activity to a level identical to the other haplotypes, for which these compounds were inactive.

Genetic variation in the **coding** region affecting the function of the enzyme may also be relevant in relation to fruit and vegetable consumption. When the detoxification function of an enzyme is affected, a higher fruit and vegetable consumption may be able to combat carcinogen load via induction of the available detoxification capacity. Alternatively, besides carcinogens, some phytochemicals are also substrates for the enzymes, and when the affinity for certain phytochemicals is affected, phytochemicals may be metabolized at a different rate.

Studies investigating the colorectal or systemic effects of fruit and vegetable consumption while taking into account polymorphisms in GSTs and NQOs have focussed on enzyme levels or activities, DNA damage or tumor occurrence (See table I) and have often investigated cruciferous vegetable intake. GSTs metabolize the cruciferous vegetable-derived bioactive isothiocyanates, hypothetically diverting them from their enzyme induction pathway to excretion. Slower metabolising genotypes would then retain these isothiocyanates longer and thus confer more protection. Urinary ITC excretion has been measured to specifically test this hypothesis. Interactions between regulatory polymorphisms and fruits and vegetables in colorectal cancer have not been studied in human populations so far.

The available study results suggest that there are differences between *GST/NQO1* genotypes with regard to associations between fruit and vegetable consumption and enzyme levels or activities, DNA damage or tumor occurrence in the **colorectal** area (Table I). There is some evidence for a lower colorectal cancer risk with higher cruciferous vegetable intake and ***GSTT1***[150, 151] or ***GSTM1*** homozygous null genotype[152], but studies measuring urinary ITC excretion do not yield consistent results supporting the mechanism of slower excretion with slower metabolising genotype[153, 154].

Table 1: Interactions between fruits/vegetables and *GST/NQO1* genotypes in colorectal cancer risk, including systemic markers

Polymorphism[a]	Exposure	Endpoint	Result[b]	Design	Reference
GSTM1	cruciferous vegetables servings/week and quartiles	**colorectal adenoma** occurrence	↓ for Q4 broccoli (not other crucifers) among *GSTM1-*	cross-sectional, 459 cases and 507 controls, USA	Lin 1998[152]
*GSTM1/T1/**P1***	330 ml juice of carrot, tomato or spinach[c], 2 weeks	lymphocyte GSTP1 protein level	↑ with carrot and tomato, independent of GST**P1** 313A>G, but more pronounced among *GSTM1+*	cross-over intervention, 23 male non-smokers, Germany	Pool-Zobel 1998[159]
		lymphocyte DNA repair capacity	↑ among *GSTM1+*		
*GSTM1/T1/**P1***	cruciferous vegetables (marker for isothiocyanates, ITCs), tertiles	urinary ITC excretion	↑ among *GSTT1+* (most pronounced among high intakes)	cross-sectional, 246 Chinese subjects, Singapore	Seow 1998[160]
GSTM1	cruciferous[d] vegetables, 6 days	serum GSTA level	↑ among *GSTM1-*, especially women	cross-over intervention, 21 men and 22 women, USA	Lampe 2000[84]
	cruciferous vegetables	serum total GST activity (NBD-Cl)	↑ among *GSTM1-*, especially women		
	cruciferous vegetables, allium[d] vegetables	lymphocyte GSTM activity	↑ among *GSTM1+*, especially women		
	apiaceous[d] vegetables	serum GSTA level	↓ among *GSTM1+* men		
	all 3 diets	serum GST activity (CDNB)	↔		
GSTM1	cruciferous vegetables + subtypes, servings/week	**colon cancer** occurrence	↔	cross-sectional, 1579 cases and 1898 controls, USA	Slattery 2000[161]
GSTT1	cruciferous vegetables + subtypes, servings/week	**colorectal adenoma** occurrence	↔	cross-sectional, 457 cases and 505 controls, USA	Lin 2002[162]
*GSTM1/T1/**P1***	cruciferous vegetables (isothiocyanates, ITCs), high/low (median-based)	**colorectal cancer** occurrence	↓ with high intake among those with *GSTM1-* àand *GSTT1-*	nested case-control, 213 incident cases and 1194 controls, Singapore	Seow 2002[150]
*GSTM1/T1/**P1**/**NQO1***	cruciferous vegetables (isothiocyanates, ITCs), quartiles	urinary ITC excretion	↑ among *GST**P1*** 313GG, ↑ among *GSTT-* (↑ with higher crucifer intake among *GST**P1*** AA but not AG/GG)	cross-sectional, 348 women, China (controls in Shanghai Breast Cancer Study)	Fowke 2003[153]
*GSTM1/T1/**P1**/**NQO1***	vegetables, cruciferous vegetables, fruit; high/low	**colorectal cancer** occurrence	↓ with high (cruciferous) vegetable intake among *GSTT1-/-* and *+/-*	cross-sectional, 484 cases and 738 controls, UK	Turner 2004[151]
GSTM1/T1	(cruciferous) vegetables	**rectal** total GST activity and GSTM1 level	↑ with high intake among *GSTM1+* genotypes	cross-sectional, 94 endoscopy patients, The Netherlands	Wark 2004[72]
GSTM1/T1	green and black tea, 4 cups/d for 4 months	DNA damage (urine)	↓ with green tea among *GSTT1+*	intervention, 143 heavy smokers, USA	Hakim 2004[163]
*GSTM1/T1/**P1***	fruits and vegetables high/low (median-based)	**colorectal cancer** occurrence	↓ with high intake among men with *GST**P1*** 313AA genotypes (especially in non-smokers and among women with *GSTT1-* genotypes)	cross-sectional, 727 cases and 736 controls, Taiwan	Yeh 2005[164]

GSTM1	standard and high-glucosinolate broccoli[e], 100 g, each 2 weeks	urinary ITC excretion	↑ among *GSTM1*-	cross-over intervention, 16 subjects, UK	Gasper 2005[154]
GSTM1	2 polyphenol-rich juices[f], each 2 weeks	leucocyte DNA damage	↔ between *GSTM1* genotypes	intervention, 27 subjects, Germany	Hofman 2006[165]
*GSTM1/T1/**A1**/**P1***	broccoli , 2.5 kg as one meal	urinary ITC excretion	↑ among *GSTM1*- ↔ between *GSTT1/**A1**/**P1*** genotypes	intervention, 88 subjects, USA	Steck 2007[155]
*GSTM1/T1/**P1**/**NQO1***	blueberry-apple juice[g] 4 weeks	lymphocyte antioxidant capacity lymphocyte DNA damage	↑ among *GSTT1*- ↓ among *GSTT1*+	intervention, 168 subjects, Netherlands	Wilms 2007[166]

[a]GSTP=313A>G, GSTA=-69C>T, GSTM1/T1=deletion polymorphisms (+ gene present, - gene absent), NQO1=609C>T, unless stated otherwise
[b]↑: endpoint higher; ↓: endpoint lower; ↔ no difference in endpoint, by genotype
[c]Pool-Zobel: juice of carrot: 21.6 β- and 15.7 α-catorotene/d; tomato: 40 mg lycopene/d, spinach: 11.5 mg lutein/d
[d]Lampe: brassica: 16 g radish sprouts, 150 g cauliflower, 200 g broccoli, 70 g cabbage; allium: 10 g chives, 100 g leeks, 5 g garlic 75 g onion; apiaceous: .50 g dill, 50 g celery, 100 g parsnips, 110 carrot
[e]:as soup, prepared in microwave oven
[f]type A: anthocyanidins-rich and type B: green tea/sioflavonoid-rich
[g]containing 97 mg quercetin and 16 mg ascorbic acid

Polymorphisms in ***GSTP1***, ***GSTA1*** and ***NQO1*** have not often been related to fruit and vegetable consumption in colorectal cancer risk and do not deliver consistent results.

Joint effects of cruciferous vegetable intake and ***GSTM1/T1*** polymorphisms have been studied in several **other cancer types** (not in table), with conflicting results, as summarized in Steck et al.[155] In a Danish nested case-control study of lung cancer there was a risk reduction of ~25% for the homozygous variant GSTP1 313GG genotype per 50% increase in vegetable consumption[156]. In a breast cancer case-control study in Shanghai, China, there was no modifying effect from cruciferous vegetable consumption between ***GSTP1*** 313A>G genotypes[157]. A large population-based breast cancer study in the USA found an increased risk among those with the homozygous variant ***GSTA1*** genotypes (*B/*B) as compared to those with the homozygous wild-type genotypes (*A/*A) in the lowest two tertiles of cruciferous vegetable consumption[158].

In conclusion: Some interactions between consumption of fruits and vegetables and GSTs and NQO1 in colorectal cancer-related endpoints have been shown. A special interest for biochemoprevention purposes lies in phytochemical-responsive SNPs in gene regulatory regions. Only recently a phytochemical-responsive promoter haplotype has been identified for GSTP1. Simultaneous assessments of polymorphisms, including phenotypic measurement in the colorectum, and thorough evaluation of genetic variation in regulatory regions has been scarce.

The following pages will summarize the rationale for the studies, state the research questions and will outline the thesis structure.

RATIONALE FOR THE STUDIES IN THIS THESIS

Promotion of total **fruit and vegetable** consumption or of subtypes such as cruciferous vegetables may be beneficial for **colorectal cancer prevention**. One hypothesized mechanism for a beneficial effect is that fruit and vegetable components, such as isothiocyanates, flavonoids, carotenoids, sulfides and phenols, can induce **biotransformation enzymes** via **regulatory DNA sequences** such as the electrophile-responsive element (EpRE), and thus increase the amount of biotransformation enzyme that is available for detoxification purposes. These detoxification enzymes then reduce the levels of carcinogens in the tissues and cells and therefore can contribute to the reduction of DNA damage and cancer risk.

GSTs and **NQO1** are common biotransformation enzymes which metabolize carcinogens relevant for the colorectal area and which have been reported to be inducible in humans by consumption of fruits and vegetables. GST levels and / or activities have been noted to be upregulated by consumption of cruciferous and allium vegetables and citrus fruits. However, results are inconsistent and usually only one fruit and vegetable subtype or GST isoenzyme is studied, most often in blood as a surrogate tissue. For NQO1 there are little data in humans. Thus, detailed GST and NQO1 **phenotype** information is needed in **colorectal tissue,** as is the consideration of several different fruit and vegetable subtypes.

Also, functional (coding) **polymorphisms** are known in GST and NQO1 detoxification enzymes, resulting in reduced enzyme function. This may reduce the capacity for detoxification of pro-carcinogens, e.g. from cigarette smoke, or prolong the presence in the body of anti-carcinogens, e.g. isothiocyanates. The specific impact of GST and NQO1 polymorphisms on colorectal enzymes or adenoma formation is not sufficiently known. Of special relevance for the colorectal cancer risk-modulating effects of fruits and vegetables are polymorphisms in **regulatory** DNA sequences of biotransformation enzymes that result in **altered responsiveness** to inducing plant food components and thereby change the availability of the biotransformation enzymes, resulting in altered protection against (pro)carcinogens. From previous research, little is known about polymorphisms in GST or NQO1 regulatory regions. Thus, an evaluation of genetic variation in regulatory regions of human GSTs and NQO1 is needed, with, if found, confirmation of functionality in cell assays and human colorectal tissue.

There are only few and inconsistent indications for interactions between consumption of fruits and vegetables and (intermediate) **cancer endpoints**.

Integrating the above, it is desirable to perform:

-a thorough evaluation of genetic variation in regulatory regions of *GSTP1*, *GSTA1* and *NQO1*

-simultaneous assessment of known polymorphisms

-further evaluation of the (combined) roles of both fruit and vegetable consumption and regulatory and coding polymorphisms in biotransformation genes in colorectal biotransformation phenotype and occurrence of colorectal tumors.

This is relevant because if functional (coding and regulatory) polymorphisms in combination with exposure to fruits and vegetables change the human GST and NQO1 detoxification enzyme level or activity, and adenoma risk, they may be important in human nutrition-related cancer prevention.

RESEARCH QUESTIONS

The overall question to be answered in this thesis is whether genetic variations in GST or NQO1 detoxification enzymes are functional in colorectal cancer prevention by fruits and vegetables, as operationalized by their enzyme phenotype and adenoma occurrence.

The following research questions were specified:

I. Enzyme phenotype (i.e. mRNA level, enzyme level, enzymatic activity level)
1) are genetic **polymorphisms** in regulatory and coding DNA sequences of GSTP1, GSTA1 and NQO1 associated with altered GST and NQO1 enzyme phenotype?
2) is recent **fruit and vegetables consumption** associated with altered GST and NQO1 enzyme phenotype?
3) does high fruit and vegetables consumption **modulate** enzyme phenotype in combination with specific genotypes (is there gene-environment interaction)?
4) is white blood cell GST and NQO1 enzyme phenotype a good **surrogate** measure for colorectal GST and NQO1 enzyme phenotype?
(chapters 2 and 4)

II. Adenoma risk
1) are genetic **polymorphisms** in regulatory and coding DNA sequences of GSTP1, GSTA1 and NQO1 associated with altered adenoma occurrence?
2) is **fruit and vegetable consumption** associated with altered adenoma occurrence?
3) is fruit and vegetable consumption associated with a different adenoma occurrence between genotypes (is there gene-environment interaction)?
(chapters 3 and 5)

Answers to these questions were sought by means of the following studies:

Genotype-phenotype study
In this observational study among 94 sigmoidoscopy patients without colorectal inflammation or cancer, *GSTA1*, *GSTP1*, *GSTM1*, *GSTT1*, *NQO1* and *NFE2L2* polymorphisms were determined, recent fruit and vegetable consumption was assessed, and GST and NQO1 enzyme levels and activities were measured in rectal biopsies.

Case-control study on colorectal adenoma risk
In this case-control study among 1477 endoscopy patients (746 adenoma cases and 698 controls), *GSTA1*, *GSTP1*, *GSTM1*, *GSTT1*, *NQO1* and *NFE2L2* polymorphisms were determined and habitual fruit and vegetable consumption was assessed.

The search for new polymorphisms and the experimental evaluation of their functionality is the subject of a related project (I.M.C.M. Rietjens, J.M.M.J.G. Aarts, A.M.J.F. Boerboom, Department of Toxicology, Wageningen University).

OUTLINE OF THE THESIS

GST enzymes are subject of chapter 2 and 3, the NQO1 enzyme is subject of chapter 4 and 5. In **chapter 2**, GST enzyme phenotypes are described in relation to *GST* genetic variation and fruit and vegetable consumption. In **chapter 3**, colorectal adenoma risk is evaluated in relation to *GST* genetic variation and cruciferous vegetable consumption. In **chapter 4**, NQO1 enzyme phenotype is described in relation to *NQO1* genetic variation and fruit and vegetable consumption. In **chapter 5**, colorectal adenoma risk is evaluated in relation to *NQO1* genetic variation, fruit and vegetable consumption and other lifestyle factors. In **chapter 6**, the results are placed in a broader context of concepts and methodology.

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ABSTRACT

High glutathione S-transferase (GST) activity may contribute to colorectal cancer prevention. Functional polymorphisms are known in the *GSTM1*, *GSTT1*, *GSTA1* and *GSTP1* genes. The influence of these GST polymorphisms and recent fruit and vegetable consumption on GST levels and activity has not been investigated simultaneously in a human population. Also, it is not clear if blood GST activity reflects rectal GST activity. Therefore, we determined GST polymorphisms in 94 patients scheduled for sigmoidoscopy. Rectal GST isoenzyme levels (GSTM1, GSTM2, GSTT1, GSTA and GSTP1) were measured by quantitative Western blotting, and rectal and white blood cell total GST activities were measured spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Vegetable and fruit consumption was assessed by dietary record. As expected, the *GSTM1* and *GSTT1* deletion polymorphisms, and the *GSTA1* g.-69C>T polymorphism significantly affected the respective isoenzyme levels. Also, rectal GST isoenzyme levels differed between those with and without recent consumption of Alliaceae, Cucurbitaceae, Apiaceae and citrus fruit. Rectal GST activity, however, was not clearly influenced by fruit and vegetable consumption. It was most significantly determined by the *GSTP1* c.313A>G polymorphism; compared to the 313AA genotypes, the 313AG and 313GG genotypes showed 36 and 67 nmol/min.mg protein ($p<0.001$) lower GST activity, respectively. The correlation between rectal and white blood cell GST activities was low ($r=0.40$, $p<0.001$), and the relevance of the various genetic and dietary factors appeared to differ between the two tissues. In conclusion, this study indicates that the GST enzyme system is influenced by both GST polymorphisms and consumption of fruits and vegetables. The latter appeared more important for individual rectal GST isoenzyme levels than for total GST activity, which could affect detoxification of isoenzyme-specific substrates. The study results do not support the use of white blood cell GST activity as a surrogate measure for rectal GST activity.

Chapter 2

Glutathione S-transferase phenotypes in relation to genetic variation and fruit and vegetable consumption in an endoscopy-based population

Mariken J. Tijhuis
Marleen H.P.W. Visker
Jac M.M.J.G. Aarts
Wilbert H.M. Peters
Hennie M.J. Roelofs
Liesbeth Op den Camp
Ivonne M.C.M. Rietjens
Anne-Marie J.F. Boerboom
Fokko M. Nagengast
Frans J. Kok
Ellen Kampman

Carcinogenesis 2007;28(4):848-57

INTRODUCTION

The gastro-intestinal tract is constantly exposed to exogenous compounds with genotoxic potential. This genotoxicity can be inherent to the compound itself or the result of endogenous bioactivation. The human body is equipped with a defense system, among which are phase II biotransformation enzymes, which alter the toxic compounds and facilitate their excretion. Phase II biotransformation enzymes have been classified into several families, based on the type of reaction they catalyze, one of the most important being the glutathione S-transferases (GSTs, EC 2.5.1.18)[1]. These enzymes catalyze the conjugation of reduced glutathione to a wide range of electrophilic substrates, including ultimate carcinogens[2, 3]. In the distal part of the gastro-intestinal tract, the colon and rectum, occurrence of neoplasia is high[4]. This may in part be due to its high cell turnover[5], but also be due to low basal GST expression[6, 7]. Support for an inverse association between GST capacity and tumor incidence is, however, mostly indirect. Other tissues, e.g. breast and lung, also show high tumor risk with relatively low GST expression and the reverse, low tumor risk with high GST expression, applies to the small intestine and liver[8]. In observational studies, lower cancer incidence has been linked to diets high in fruits and vegetables[9]. An explanation for this observation may be that fruit and vegetable components, e.g. from Brassica vegetables and citrus fruits, induce detoxification enzymes such as GSTs[10]. In a small number of human dietary intervention studies GSTs have been reported to be inducible, in various tissues; in the colorectal area[11, 12], in plasma or peripheral lymphocytes[13-16], and in saliva[16, 17]. Because GST levels are relatively low in the colorectal area as compared to other organs, upregulation of GST-enzymes may have considerable protective impact in the colon and rectum[6, 7]. The induction process involves activation of certain signal transduction pathways by fruit and vegetable components acting through transcription factor binding sites which are present in the promoters of GSTs[18].

Interestingly, interindividual polymorphic variation exists in the GST genes[3]. Depending on exposure to (pro)carcinogens, individuals with different genetic GST variants have been reported to have moderately different cancer risks[3], adding more support for a role of GSTs in human cancer susceptibility. In the coding sequence of GST genes, genetic polymorphisms can affect e.g. the catalytic activity of the enzymes[19]. In the regulatory sequence, genetic variation can result in altered binding of transcription factors and altered mRNA levels[20], translating into changes in GST isoenzyme levels[21]. In light of the effects that fruit and vegetable components may have on the regulatory region, polymorphisms in this area could be important for nutritional strategies aiming to upregulate GST enzyme capacity.

The aim of the present human observational study was to comprehensively assess GST protein phenotypes (i.e. GST isoenzyme levels and total GST activity) in the rectum in relation to genetic variation (i.e. *GSTM1*, *GSTT1*, *GSTA1* and *GSTP1* polymorphisms) and recent consumption of fruits and vegetables. Moreover, white blood cell GST activities were measured to investigate if these factors show similar effects on blood GST phenotype, and thus if blood can be used as a surrogate tissue.

METHODS

Population. Participants were recruited in two outpatient endoscopy clinics in the Netherlands from patients scheduled for a sigmoidoscopy, between January 2003 and June 2004. Eligibility criteria were: age between 18 and 75 years, Caucasian, no chronic inflammatory bowel disease (past or present), no inflammation in the distal colon at the time of endoscopy, no sporadic colorectal cancer (past or present), and no bowel resection. Of all eligible patients, 235 (64%) were invited to participate in the study. Invitation was dependent on whether the time between identification of the patient and the start date of the food record was sufficient for the consent procedure. Of all invited patients, 105 agreed to participate (45%). Main reasons for not participating were the stress caused by the prospect of the endoscopy in general and fear for the biopsy specifically. Of all participants, 11 were excluded at endoscopy or after inspection of tissue pathology (PA) results because inclusion criteria were not met. This resulted in a final study population of 94 individuals. The study was approved by the Medical Review Boards of both hospitals. All participants gave their written informed consent.

Medical, dietary and lifestyle information. The indication for endoscopy was recorded from the endoscopy request form. Information on the macroscopic and (if available) microscopic result of the endoscopy was recorded from the endoscopy report and (if available) PA report.

Participants kept a 3-day dietary record, the third day ending at the time of endoscopy. Type and amount of food consumed were recorded in a structured open entry format. All records were checked for quality and completeness by the same, trained dietician of the division of Human Nutrition of Wageningen University; about 50% of dietary records required follow-up by telephone, which was successful for the majority within 3 days after endoscopy. Processing into food quantities and coding was done according to the most recent standard manual on food portions and household measures and the Dutch Food composition Table[22, 23]. Conversion into amounts of nutrients was done using the VBS Food Calculation System[24]. Fruits were subdivided in citrus (fruit and juice) and non-citrus fruits,

vegetables in botanically defined subtypes: Alliaceae (e.g. garlic, leek), Apiaceae (e.g. celery, carrot), Brassicaceae (e.g. cauliflower, broccoli), Compositae (e.g. endive, lettuce), Cucurbitaceae (e.g. zucchini, cucumber), Solanaceae (e.g. bell pepper, tomato; potato not included), and a restgroup.

General lifestyle information was collected through a semi-structured questionnaire containing questions about age, sex, weight, height, smoking habits, medication, disease and family history of cancer.

Specimen collection and preparation. *Biopsies.* Flexible sigmoidoscopy was performed with the patient in left lateral decubitus position. Biopsies (approximately 20 mg each) were taken from normal rectal mucosa at a distance of 5 to 15 cm from the anal verge and were snap-frozen in liquid nitrogen. *Blood.* Blood (3 x 9 ml) was drawn shortly after endoscopy by venipuncture in Vacuette EDTA K3 Tubes (Greiner Bio-One, Alphen a/d Rijn, the Netherlands). *Leukocytes and lymphocytes*. Within 5 min after blood draw, duplicate portions of 1.4 ml whole blood were mixed with 12.6 ml Puregene RBC lysis solution (Gentra systems, BIOzym group, Landgraaf, The Netherlands) and kept on ice for 10-30 minutes. Samples were then centrifuged at 4°C and 2500 g for 10 min. Leukocyte pellets were not collected of the first 19 participants. Duplicate portions of 4 ml of whole blood were used for isolation of lymphocytes on Histopaque-1077 (Sigma-Aldrich, Zwijndrecht, The Netherlands) according to the instruction of the manufacturer. Leukocyte and lymphocyte pellets were resuspended in 1 ml phosphate buffered saline (PBS, pH 7.4, Invitrogen, Breda, The Netherlands), centrifuged for 5 minutes at 10,000 g and stored at -80°C. *Leukocyte DNA*. One tube of whole blood was centrifuged at 1100 g for 10 min. After removal of plasma, the buffy coat layer was remixed with the blood remnant for later DNA extraction and stored at -80°C.

Laboratory analyses. *Genotyping*. DNA was extracted from buffy coat cells (QIAamp 96 DNA blood kit, Qiagen Benelux B.V., Venlo, The Netherlands), and samples were stored with negative controls at 4°C. The *GSTM1* and *GSTT1* deletion polymorphisms were determined simultaneously by allele-specific multiplex PCR[25], in which a ß-globin gene fragment was co-amplified as internal positive control. The *GSTA1* g.-69C>T polymorphism was determined by PCR-RFLP according to Coles et al.[26]. Some modifications to the Coles' protocol were made; annealing temperature was set at 61°C and *Eam*1104I (Fermentas GmbH, St. Leon-Rot, Germany) was used as restriction enzyme. The *GSTP1* c.313A>G polymorphism was assessed by PCR-RFLP according to Harries et al.[27]. *GSTA1* g.-69C>T and *GSTP1* c.313A>G genotypes were in Hardy Weinberg Equilibrium (HWE; χ^2=2.37, p-value=0.12 and χ^2=0.07, p-value=0.79, respectively). The 5' regulating region of the *GSTP1* gene was screened for new polymorphisms by

DNA sequencing of the -434 to +296 region of the *GSTP1* gene (relative to the translation initiation site of GenBank Accession AY324387; experimental details available on request). No new polymorphisms were identified. We confirmed the *GSTP1* g.217G>A (rs1079719 on the NCBI SNP website), g.227G>A (rs1871041) and g.272C>G (rs4147581) polymorphisms and genotyped them by pyrosequencing (the g.223 G-insertion polymorphism was also confirmed, but genotyping was unsuccesful). For genotyping, PCR was performed with AccuPrime GC-rich DNA polymerase (Invitrogen). The resulting 279 bp amplicon was used for two reverse directed pyrosequencing analyses; the first to analyze the 233 to 209 region of *GSTP1* in order to genotype the g.217G>A and g.227G>A polymorphisms and the second to analyze the 275 to 266 region of *GSTP1* in order to genotype the g.272C>G polymorphism. The *GSTP1* g.217G>A and g.227G>A genotypes were in HWE (χ^2=0.15, p=0.70 and χ^2=1.74, p=0.19, respectively), whereas the *GSTP1* g.272C>G polymorphism was not (χ^2=5.58, p=0.018). All polymorphisms were genotyped in duplicate; reproducibility was 100%.

Protein assays. Two rectal biopsies were homogenized on ice using a frozen (-20°C) pestle, and suspended in 100 µl of 20 mM Tris-HCl (pH 7.5). Leukocyte and lymphocyte pellets were resuspended in 100 µl 20 mM Tris-HCl and cells were lysed by sonification (Sonorex RK100 ultrasonic bath, Bandelin electronic, Berlin, Germany) on ice during 10 min. The resulting rectal tissue and white blood cell lysates were centrifugated at 16,000 g and supernatants were alliquoted and refrozen at -80°C, until further measurement.

Total protein was measured by the BCA protein assay reagent kit (Pierce Rockford, IL, USA) using BSA as a standard, following the manufacturer's instructions.

Levels of rectal GST M1, M2, T1, A and P1 were determined by western blotting using monoclonal antibodies[11] and subsequent densitometric analyses of immunoblots. Known amounts of purified GSTs were run in parallel with the samples and served as standards. For quantification of GSTM2 protein, M2 bands were calculated relative to the M1 standard. The detection limit of the immunoblot assays was 20 ng GST protein/mg total protein. GST isoenzyme levels were was normalized to total protein content and expressed as ng GST protein/mg total protein.

Total GST enzyme activity was measured spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich) as a substrate according to the method of Habig et al.[28], but adapted for microplate reader (SpectraMax 340, Molecular Devices Corporation)[29]. Measurements were performed at 340 nm and 37°C, for 3 minutes, in triplicate. Data were analyzed using SOFTmaxPRO software

(version 2.2.1, Molecular Devices Corporation). The average coefficient of variation in GST activity was 7.3% for rectal samples, 12.4% for leukocytes and 11.2% for lymphocytes. GST enzyme activity was expressed as nmol 2,4-dinitrophenyl-S-glutathione (DNP-SG) produced/min.mg total protein. *Sample storage*. White blood cell pellets were stored intact at -80°C for 6.3±4.5 months after blood sampling until preparation and refreezing. GST activity was then measured within 1 month. Rectal biopsies were stored intact at -80°C for 7.5±4.5 months after tissue sampling until homogenation and refreezing; GST activity was then measured within 1 month and GST isoenzyme levels ~3 months later.

Statistical analyses. From two subjects no rectal tissue was obtained and for another two subjects there was no dietary information available. One extreme outlier in lymphocyte GST activity (523 nmol/min.mg protein) was excluded from lymphocyte-analyses. For two subjects with the *GSTM1* null genotype, a GSTM1 protein value was measured. Since some cross-reactivity with other GSTM proteins may have occurred here, these two GSTM1 values were set to zero.

Vegetable and fruit consumption was dichotomized as did or did not consume on one of the two days before endoscopy. Linear regression models were used to evaluate factors confounding the associations between genotype and phenotype, or between fruit and vegetable consumption and phenotype (i.e. >10% change in β-estimate), or statistically significantly contributing to phenotype (i.e. $p<0.05$). GST genotype-phenotype associations were evaluated for age, sex, sample storage time and family history of colorectal cancer, and models were adjusted for age, sex and sample storage time. Sample storage time was calculated as the time between specimen collection and measurement performance, and contributed statistically significantly to phenotype in most models. Although age and sex did not contribute significantly, they were included because they are important general population parameters. Fruits and vegetables-GST phenotype associations were evaluated for age, sex, sample storage time, outpatient clinic, family history of colorectal cancer, season, smoking, coffee consumption, alcohol consumption, presence of diverticula, hemorrhoids, and adenomas, and models were adjusted for age, sex, sample storage time and smoking. Smoking was defined as smoking on one of the two days before endoscopy.

The difference in GST phenotype outcome and its 95% confidence interval was reported for the variant genotypes as compared to the most common homozygous genotype variant, and for the 'did-consume' groups as compared to the non-consumers. For the reference groups (the most common homozygous genotype variant and the non-consumers), least-squares adjusted means were calculated, using mixed models.

Pearson correlation coefficients were calculated to evaluate the strength of the association between rectal and white blood cell GST activities.

Haplotypes for *GSTP1* polymorphisms were estimated using the Hplus program version 2.5, available online (Fred Hutchinson Cancer Research Center. Hplus. http://qge.fhcrc.org/hplus). The *GSTP1 g.*217G>A, g.227G>A and g.272C>G variant nucleotides were not linked and were therefore investigated separately. Inclusion of the *GSTP1* c.313A>G polymorphism (corresponding to genomic position 1377) in haplotype estimation resulted in 6 haplotypes, the two most common were: GG*G*-A (44.2%) and *A*GC-*G* (30.5%). All statistical analyses were performed using SAS software, version 9.1 (SAS institute, Cary, NC).

RESULTS

Table 1 shows the distribution of general characteristics and consumption of fruit and vegetable subtypes between groups of lower and higher rectal GST activity within the study population. Rectal GST activity was lower with higher age, longer duration of ex-smoking, and the presence of hemorrhoids and diverticula. In the higher rectal GST activity group, there were more Alliaceae consumers.

In table 2 the GST genotypes and phenotypes are presented for the rectal GST activity groups and for the total population. Rectal GST activity was higher with presence of the *GSTP1* 272G- and 313A-alleles. All GST isoenzyme levels were higher with higher total rectal GST activity, GSTM2 and GSTP1 most pronounced. GSTP1 was the most abundant rectal GST enzyme measured, GSTA the least abundant. Interindividual variation in GST M1, M2, T1, A and P1 isoenzyme levels was about 10, 15, 7, 17 and 13-fold, respectively. Total GST activities in leukocytes and lymphocytes were higher with higher total rectal GST activity, though not significantly in lymphocytes. The interindividual variation in the GST activity measures was about 4-fold. The correlations between rectal and white blood cell GST activities were 0.41 between rectal and leukocyte, 0.35 between rectal and lymphocyte, and 0.41 between leukocyte and lymphocyte GST activity, $p<0.001$ for all coefficients.

Rectal GST isoenzyme levels in relation to genetic variation. GST polymorphisms were associated with the levels of their isoenzymes (not in table); presence or absence of the *GSTM1* gene resulted in GSTM1 levels of 2150±1148 and 0 ng/mg protein and for the *GSTT1* gene corresponding GSTT1 levels were 4646±2466 and 0 ng/mg protein. GSTA level differed significantly between *GSTA1* g.-69C>T genotypes (Figure 1); The CC genotypes had the highest (762±413 ng/mg, median value 677), the CT genotypes an intermediate (378±264 ng/mg, median value 368) and the TT genotypes no detectable GSTA level ($p<0.001$). The GSTP1

level appeared to be higher among individuals with a *GSTP1* 313 variant G-allele: the 313GA and 313GG genotypes had 772 (p=0.17) and 1440 (p=0.088) ng/mg protein higher GSTP1 levels compared to the AA genotype, respectively. The *GSTP1* 217GA genotype also showed a borderline significantly higher GSTP1 level (1026 ng/mg, p=0.072) compared to the 217 GG genotype. There was no association between GSTP1 level and the *GSTP1* 227 or 272 variants.

Table 1: General study population characteristics by rectal GST activity

Characteristic		Rectal total GST activity (nmol DNP-SG/min.mg protein)[a]			
		≤ median, n=46		>median, n=46	
General characteristics		*mean±sd or n*			
Age	years	51±14		42±11**	
Sex	male	20		19	
Education	low/high	9, 19		7, 21	
Smoking	never	17		19	
	current/ ex	6, 23 (quit: 19±11 yrs)		12, 15 (quit: 10±10 yrs)*	
BMI		25.3±4.4		26.0±4.8	
Medical factors[b]		*n*			
Family history	cancer (colorectal)	27 (2)		20 (4)	
Indication for endoscopy[c]	blood loss per anum	21		23	
	abdominal pain	15		15	
	different defecation pattern	15		14	
Endoscopy characteristics[d]	outpatient clinic (A/B)	41/5		36/10	
	morning/afternoon	24/22		24/22	
	hemorrhoids	13		5*	
	diverticula	11		3*	
	adenoma(s)	3		8	
Intake of fruit and vegetable subtypes[e]		*p50 (p25-p75); no. of consumers*			
Vegetables, g/day	total[f]	117 (77-168)	40	100 (51-219)	39
	Alliaceae	11 (5-38)	13	27 (15-33)	23*
	Apiaceae	33 (5-50)	10	49 (12-110)	15
	Brassicaceae	43 (22-86)	16	40 (24-82)	13
	Compositae	38 (23-75)	21	39 (19-90)	16
	Curcubitaceae	47 (25-51)	16	30 (22-75)	13
	Solanaceae[f]	40 (29-95)	16	34 (18-55)	14
	other	60 (17-109)	8	80 (73-101)	9
Fruits, g/day	total	138 (70-265)	34	85 (44-185)	36
	citrus	68 (55-120)	14	55 (30-131)	12
	citrus juice	203 (88-303)	12	125 (60-165)	7

[a]median value on which groups are based: 214 nmol/min.mg protein

[b]source: medical record and self-reported

[c]most common; more than one possibility per individual

[d]bowel preparation, day before: 17:00 hrs: 2 laxatives (10 mg), 20:00 hrs microlax enema (5 ml), in clinic B 2x different preparation

*<0.05; **<0.001 (χ^2 or fisher depending on cell numbers for proportions; wilcoxon for non-normally distributed variables; t-test for normally distributed variables)

Table 2: GST genotype and phenotype characteristics of the total study population and by rectal GST activity

	Rectal total GST activity (nmol DNP-SG /min.mg protein)[a]		
	≤ median, n=46	> median, n=46	total population
Genotyping results	n		%
GSTM1 gene deletion (gene present/absent)	20 / 26	23 / 22	48 / 52%
GSTT1 gene deletion (gene present/absent)	34 / 12	39 / 7	89 / 21%
GSTA1 g.-69>T (CC/CT/TT)	20 / 23 / 3	11/ 28 / 7	33 / 55 / 12%
GSTP1 g.217G>A (GG/GA/AA)	13 / 26 / 7	19 / 20 / 7	34 / 51 / 15 %
g.227G>A (GG/GA/AA)	33 / 10 / 3	38 / 8 / 0	78 / 19 / 3%
g.272C>G (CC/CG/GG)	13 / 30 / 3	8 / 27 / 11*	22 / 63 / 15%
c.313A>G (AA/AG/GG)[b]	13 / 25 / 8	25 / 18 / 3*	41 / 47 / 12%
Enzyme measurements[c]			
GST isoenzyme level, rectum		ng/mg protein	
M1	929 ± 1354	1091 ± 1311	1010 ± 1328
M2	1119 ± 598	1533 ± 623**	1326 ± 642
T1	3221 ± 2707	4152 ± 3031	3647 ± 2906
A	427 ± 265	505 ± 485	473 ± 388
P1	5584 ± 2264	7300 ± 2510***	6442 ± 2529
GST activity		nmol DNP-SG/min.mg protein	
rectum	173 ± 30[a]	258 ± 47[a]	216 ± 58
leukocytes	118 ± 32	146 ± 39*	134 ± 38
lymphocytes[d]	119 ± 28	131 ± 31	125 ± 30

[a]median value on which groups are based: 214 nmol/min.mg protein

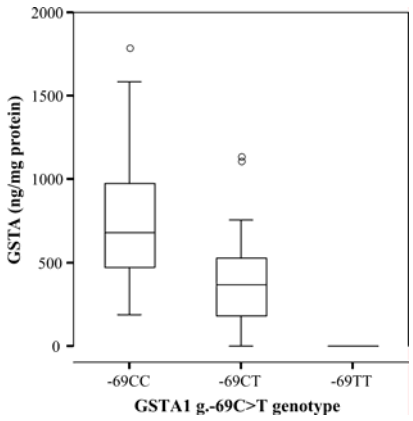
[b]corresponds to genomic position 1377

[c]for rectal tissue, sample storage time until homogenation and refreezing was 9.4±5.0 for lower than and 5.6±3.0 months for higher than median GST activity; for white blood cells this was 8.2±5.0 for lower than and 4.4±3.0 months for higher than median GST activity

[d]one outlier deleted

*<0.05; **<0.01, ***<0.001 (χ^2 or fisher depending on cell numbers for proportions; wilcoxon for non-normally distributed variables; t-test for normally distributed variables)

Figure 1: Rectal GSTA level by *GSTA1* g.-69C>T genotype



Footnote:
Boxes and bars represent the interquartile range and the median value, respectively; Whiskers represent distance to the smallest and largest sample value, excluding outliers; open circles represent values of 1.5-3 box lengths.

Table 3: Rectal GST isoenzyme levels (ng/mg protein) by recent fruit and vegetable consumption (yes or no)

Consumption[c]		n[a]	GSTM1		GSTM2		n[a]	GSTT1		n[b]	GSTA		GSTP1	
		43	reference mean* +/- difference (95% CI)	p-value	reference mean* +/- difference (95% CI)	p-value	72	reference mean* +/- difference (95% CI)	p-value	90	reference mean* +/- difference (95% CI)	p-value	reference mean* +/- difference (95% CI)	p-value
Total fruit	no	8	2585		1371		15	5118		20	476		6591	
	yes	35	-703 (-1671, 266)	0.15	-19.9 (-355, 315)	0.91	57	-529 (-2089, 1032)	0.50	72	+77.7 (-126, 282)	0.45	+592 (-698, 1881)	0.36
citrus juice+fruit	no	26	1722		1317		43	4658		52	525		7102	
	yes	17	+826 (42.5, 1610)	0.039	+109 (-169, 388)	0.44	29	+250 (-1083, 1582)	0.71	40	+7.59 (-163, 178)	0.93	-311 (-1389, 767)	0.57
Total vegetables	no	7	2451		1850		9	4377		12	562		8029	
	yes	36	-460 (-1580, 660)	0.41	-570 (-976, -164)	0.007	63	+408 (-1651, 2467)	0.69	80	-39.5 (-299, 220)	0.76	-1205 (-2824, 415)	0.14
Alliaceae	no	24	2107		1312		46	4178		56	513		6455	
	yes	19	-80.6 (-883, 721)	0.84	+106 (-175, 388)	0.45	26	+1431 (112, 2751)	0.034	36	+35.8 (-136, 208)	0.68	+1262 (206, 2318)	0.020
Apiaceae	no	30	2225		1438		53	4607		66	540		7154	
	yes	13	-497 (-1322, 329)	0.23	-338 (-631, -46.0)	0.024	19	+572 (-834, 1978)	0.42	26	-48.7 (-232, 135)	0.60	-693 (-1849, 462)	0.24
Brassicaceae	no	32	1927		1333		47	4545		63	537		7075	
	yes	11	+595 (-229, 1419)	0.15	+79.8 (-210, 370)	0.59	25	+645 (-676, 1967)	0.33	29	-29.8 (-207, 147)	0.74	-289 (-1409, 831)	0.61
Cucurbitaceae	no	27	2020		1335		51	4744		62	520		6698	
	yes	16	+154 (-629, 938)	0.69	+84.3 (-209, 378)	0.57	21	-0.04 (-1394, 1394)	1.00	30	+31.6 (-148, 211)	0.73	+1105 (-6.40, 2216)	0.051

*adjusted for age, sex, sample storage time, smoking

[a]n: only those who possess the gene

[b]n is the same for GSTP1, GSTA and GSTM2, i.e. total population

[c]on (one of) the two days before specimen collection

Rectal GST isoenzyme levels in relation to fruit and vegetable consumption. Citrus fruit and juice consumption combined was positively associated with GSTM1 level (Table 3). Total vegetable consumption and consumption of Apiaceae were inversely associated with GSTM2 level. Consumption of Alliaceae was associated with higher GSTT1 level. There was no significant association between fruit or vegetable consumption and GSTA level. However, when the *GSTA1* g.-69C>T genotype was added to the GSTA-fruit and vegetable models, GSTA level was significantly higher among consumers of Solanaceae: +152 ng/mg protein (p=0.024), and Cucurbitaceae: +152 ng/mg protein (p=0.035), compared to non-consumers (data not shown in table). Consumption of Alliaceae and Cucurbitaceae was positively associated with GSTP1 level (Table 3). This higher GSTP1 level was most pronounced for the *GSTP1* 272CC genotype: +3758 ng/mg protein (p=0.0039) with Alliaceae consumption and +2340 ng/mg protein (p=0.074) with Cucurbitaceae consumption; similar results were observed for the 227GA genotype, but this did not reach statistical significance. There was no such difference when stratifying for the *GSTP1* g.217G>A or c.313A>G genotypes.

Rectal and white blood cell total GST activity in relation to genetic variation. *Rectal GST activity.* The polymorphisms in *GSTM1*, *GSTT1* and *GSTA1* did not affect rectal GST activity significantly (Table 4). Rectal GST activity did differ significantly between *GSTP1* c.313A>G genotypes; the 313AG and GG genotypes had 36 and 67 nmol/min.mg protein lower rectal GST activities than the AA genotype, respectively. The *GSTP1* 272GG genotypes showed significantly higher rectal GST activity, likely because all 272GG individuals were *GSTP1* 313AA. The *GSTP1* g.217G>A and g.227G>A polymorphisms did not affect rectal GST activity significantly.

White blood cell GST activity. In leukocytes, the *GSTM1* deletion polymorphism was associated with a 28 nmol/min.mg protein lower GST activity (Table 4). As in rectum, the GSTP1 313G-variant showed lower activity in leukocytes, but this was not statistically significant. The *GSTP1* 272CG genotype was significantly inversely associated with leukocyte activity, which was in contrast to its effect on rectal GST activity. In lymphocytes, the *GSTP1* c.313A>G polymorphism showed a similar trend as in rectum: the 313AG and 313GG genotypes had 17 and 35 nmol/min.mg protein lower GST activity than the 313AA genotype, respectively. The *GSTP1* 217GA genotypes had significantly lower and the *GSTP1* 227GA genotypes had significantly higher lymphocyte GST activities than their homozygous wildtypes (*GSTP1* 217GG and 227GG, respectively).

Table 4: Rectal and white blood cell GST activity (in nmol DNP-SG/min.mg protein) by GST genotype

		Rectal total GST activity			White blood cell total GST activity					
					Leukocytes			Lymphocytes		
Genotype		n	reference mean* +/- difference (95% CI)	p-value	n	reference mean* +/- difference (95% CI)	p-value	n	reference mean* +/- difference (95% CI)	p-value
		92			75			93[a]		
GSTM1 deletion	gene present	43	218		34	149		45	128	
	gene absent	49	-4.4 (-27, 19)	0.71	41	-28 (-44, -11)	0.001	48	-3.8 (-16, 8.7)	0.55
GSTT1 deletion	gene present	73	219		61	135		74	126	
	gene absent	19	-12 (-40, 16)	0.39	14	-3.2 (-26, 19)	0.78	19	-0.61 (-16, 15)	0.94
GSTA1 g.-69C>T	CC	31	201		22	127		31	127	
	CT	51	+23 (-1.2, 48)	0.062	42	+11 (-9.0, 31)	0.28	51	-1.9 (-16, 12)	0.79
	TT	10	+28 (-12, 68)	0.16	11	+6.9 (-21, 35)	0.63	11	-6.0 (-28, 16)	0.58
GSTP1 g.217G>A	GG	32	230		22	139		31	135	
	GA	46	-21 (-46, 3.9)	0.098	42	-12 (-32, 7.7)	0.23	48	-14 (-28, -0.43)	0.043
	AA	14	-23 (-58, 11)	0.18	11	+13 (-14, 41)	0.34	14	-13 (-32, 5.7)	0.17
GSTP1 g.227G>A	GG	71	216		62	130		73	123	
	GA	18	+10 (-18, 38)	0.48	12	+20 (-3.2, 44)	0.090	17	+17 (1.2, 33)	0.036
	AA	3	-61 (-124, 2.8)	0.061	1	+44 (-33, 121)	0.26	3	-0.67 (-36, 34)	0.97
GSTP1 g.272C>G	CC	21	200		15	153		21	126	
	CG	57	+14 (-13, 41)	0.30	49	-24 (-46, -2.2)	0.032	58	-3.3 (-18, 12)	0.66
	GG	14	+45 (8.4, 82)	0.017	11	-20 (-49, 9.4)	0.18	14	+12 (-8.2, 33)	0.24
GSTP1 c.313A>G	AA	38	241		27	144		38	138	
	AG	43	-36 (-59, -13)	0.002	41	-16 (-35, 2.8)	0.094	44	-17 (-30, -4.8)	0.007
	GG	11	-67 (-100, -33)	0.000	7	-13 (-45, 19)	0.48	11	-35 (-54, -17)	0.000

*adjusted for age, sex, sample storage time
[a]1 outlier deleted

Rectal and white blood cell total GST activity in relation to fruit and vegetable consumption. *Rectal GST activity.* There was no statistically significant association between fruit and vegetable consumption on the two days prior to sigmoidoscopy and rectal GST activity (Table 5). However, when stratifying for the GSTM1 deletion polymorphism, there was a significant difference in rectal GST activity between those who did and did not report fruit consumption of any kind among *GSTM1* null individuals (+43 nmol/min.mg protein, p=0.019, data not in table), which was not observed among *GSTM1* positive individuals (-1.5, p=0.95). Also, when adding the *GSTP1* c.313A>G polymorphism to the rectal GST activity and fruit and vegetable models, there was a significant difference between consumers and non-consumers of Alliaceae with respect to total rectal GST activity (+24 nmol/min.mg protein, p=0.031).

White blood cell GST activity. Consumption of citrus juice was slightly inversely associated with leukocyte GST activity, and consumption of Alliaceae and Cucurbitaceae positively (Table 5). Lymphocyte GST activity appeared to be slightly positively associated with citrus fruit consumption.

Similar to observations in rectal biopsies, lymphocyte GST activity among GSTM1 null individuals who reported fruit consumption was higher compared to those who did not (+18, nmol/min.mg protein p=0.068), and this was not observed among GSTM1 positive individuals (-16 nmol/min.mg protein, p=0.28); In leukocytes, GST activity among GSTM1 null and positive individuals with fruit consumption was +17 (p=0.13) and -5.1 (p=0.76), respectively. The difference in rectal GST activity, observed with Alliaceae consumption when the GSTP1 c.313A>G polymorphism was added to the statistical model, could also be seen in lymphocytes: +13 nmol/min.mg protein (p=0.045) and became slightly more pronounced in leukocytes: +17 nmol/min.mg protein (p=0.076).

Table 5: Rectal and white blood cell total GST activity (in nmol DNP-SG/min.mg protein) by recent fruit and vegetable consumption (yes or no)

		Rectal total GST activity			White blood cell total GST activity					
					Leukocytes			Lymphocytes[a]		
Consumption[b]		n	reference mean[*] +/- difference (95% CI)	p-value	n	reference mean[*] +/- difference (95% CI)	p-value	n	reference mean[*] +/- difference (95% CI)	p-value
		90			73			91		
Total fruit	no	20	209		19	133		20	124	
	yes	70	+22 (-5.5, 50)	0.12	54	+5.7 (-15, 26)	0.58	71	+5.4 (-12, 22)	0.51
Citrus fruit	no	64	223		51	136		65	125	
	yes	26	+5.8 (-21, 32)	0.66	22	+0.61 (-20, 22)	0.95	26	+13 (-2.2, 27)	0.095
Citrus juice	no	71	227		54	140		70	129	
	yes	19	-17 (-45, 10)	0.21	19	-18 (-37, 1.0)	0.063	21	-9.3 (-24, 5.8)	0.23
Total vegetables	no	11	222		10	135		12	119	
	yes	79	+2.9 (-33, 39)	0.88	63	+0.93 (-26, 28)	0.95	79	+9.5 (-10, 29)	0.34
Alliaceae	no	54	218		41	129		56	124	
	yes	36	+15 (-9.0, 38)	0.22	32	+16 (-2.3, 33)	0.086	35	+7.5 (-6.1, 21)	0.27
Apiaceae	no	65	226		51	135		65	125	
	yes	25	-5.8 (-31, 20)	0.65	22	+2.2 (-17, 21)	0.82	26	+8.5 (-5.8, 23)	0.24
Brassicaceae	no	61	228		46	136		62	128	
	yes	29	-11 (-36, 13)	0.36	27	-0.65 (-19, 18)	0.94	29	-2.6 (-17, 11)	0.71
Cucurbitaceae	no	61	222		49	131		62	126	
	yes	29	+8.2 (-17, 33)	0.52	24	+18 (-0.10, 37)	0.051	29	+5.7 (-8.4, 20)	0.42

[a]one outlier deleted

[b]on (one of) the two days before specimen collection

[*]adjusted for age, sex, sample storage time, smoking

DISCUSSION

The results from this human observational study confirm that both genetic variation and recent consumption of fruits and vegetables influence the GST enzyme system. The *GSTM1, GSTT1* and *GSTA1* g.-69C>T polymorphisms clearly affected their respective isoenzyme levels. Consumption of specific fruits and vegetables was also associated with differences in specific rectal GST isoenzyme levels; GSTM1 level was higher among consumers of citrus, GSTM2 level was lower among total vegetable and Apiaceae consumers, GSTT1 level was higher among consumers of Alliaceae, and GSTP1 level was higher among consumers of Alliaceae and Cucurbitaceae. The consequences for the rectal GST activity are not obvious; activity was most affected by the *GSTP1* c.313A>G polymorphism, whereas fruit and vegetable consumption in itself did not seem influential. GST activity in white blood cells did appear to be affected by fruit and vegetable consumption to some extent, and next to *GSTP1* polymorphisms also by the *GSTM1* deletion genotype.

We will first discuss the polymorphisms, and then fruit and vegetable consumption, in relation to GST phenotype.

In our population, the *GSTA1* g.-69C>T polymorphism affected rectal GSTA enzyme level, which is consistent with knowledge about the functionality of the polymorphism[30]. GSTA level as measured by us, consists of GSTA1 and GSTA2 protein. As we observed GSTA levels of zero (among the *GSTA1* -69TT-genotypes), the expression of the GSTA2 subunit was apparently below detection in these subjects. Possibly, the reversed co-expression between GSTA1 and GSTA2, as reported for liver[26], does not operate in the colorectal area. The *GSTP1* c.313A>G polymorphism did not have a clear relationship with its enzyme level, but clearly affected rectal total GST activity. It leads to an amino acid change, with consequences for substrate binding and thermal stability, i.e. lower GSTP1 activity[19, 31]. The abundance of GSTP1 enzyme in the rectum, in combination with the lower activity of the variant GSTP1 protein, explains the great influence of the *GSTP1* c.313A>G polymorphism on total GST activity in the rectum. The effect of the other *GSTP1* polymorphisms may be related to the *GSTP1* c.313A>G polymorphism, as the 313A-allele occurred most frequently with the 272G-allele, and the 313G-allele with the 217A-allele. However, a larger population should be genotyped for more definitive answers concerning their linkage.

Consumption of different types of fruits and vegetables appeared to influence rectal individual GST isoenzyme levels, while it did not seem to influence rectal total GST activity. In the literature, several GST protein phenotypes and fruit and vegetable subtypes have been investigated, in different tissues. Most studies have

focussed on Brassicaceae[11, 13, 14, 16, 17, 32-34]: higher levels of GSTA[11, 13-15] and GSTP1[11], and higher total GST activity[17] and GSTM activity[15] have been reported. We observed higher GSTM1 levels in rectum among Brassicaceae consumers (~30%, not significant), but a significant positive effect of Brassicaceae consumption on GST isoenzyme levels or GST activity was not seen. Lampe et al. also studied Alliaceae and Apiaceae vegetables; After Alliaceae supplementation, they observed higher GSTM activity[15]. In our study, individuals who consumed Alliaceae had significantly higher GSTT1 and GSTP1 level. The latter is in contrast with Wark et al. who, also from observational data, noted downregulation of rectal GSTP1 with higher Alliaceae consumption[35]. After Apiaceae supplementation, Lampe et al. observed lower serum GSTA levels[15]. They have also reported decreased CYP1A2 activity with Apiaceae consumption[36]. In our study, rectal GSTM2 appeared to be downregulated by Apiaceae. There are indications from cell studies that phytochemicals from Apiaceae and Brassicaceae downregulate certain enzymes[37, 38]. Also, we observed lower GSTP1 levels with total vegetable consumption, to a level of about 85% of those who did not consume any vegetables on one of the two days before endoscopy; This effect appeared to be due to Apiaceae and Brassicaceae vegetables. A similar decrease in GSTP1 level was noted in lymphocytes by Persson et al. after a mixed vegetable diet, containing Apiaceae and Brassicaceae[39]. Overall, inconsistencies concerning the effect of fruit and vegetable consumption on GST phenotype remain. Differences in study results may be related to study design (intervention versus observational), time frame and method of food consumption measurement (food frequency questionnaire versus food record), coding of consumption (yes/no vs high/low or continuous), type of tissue that was sampled, or laboratory methods. In general, studies are small, and only few have taken genetic variation into account.

Our a priori calculation of power and sample size required for comparison of phenotype by genotype groups was based on data by Coles et al.[26] and Siegel et al.[40]. We estimated that a total group of 100 subjects and differing genotype frequencies would yield a power of >75% to detect relevant protein differences between at least two of the three genotype groups. To uncover the interplay between several different exposures and genes, however, larger studies are needed. As sampling of rectal material is invasive and impractical, blood GST activity could be a useful surrogate measure. We therefore compared white blood cell and rectal GST activity, but the correlation between the two was low. Despite earlier reports[33, 41], blood may not be a good surrogate tissue. Possibly, taking genotype into account improves its value.

The study population consisted of individuals undergoing sigmoidoscopy for diagnostic reasons. Possible disadvantages are presence of colorectal abnormalities, bowel preparation, and lower food consumption. Colorectal abnormalities (i.e. diverticula, hemorrhoids, and adenomas) were minor however, and though their distribution over the GST activity median-based groups was different, they did not influence statistical models. Bowel preparation for endoscopy may have affected phenotype, by itself or by lowering absorption and delivery of (non)nutrients to the rectal crypts. If it did, subjects at least were affected similarly, as preparations were similar. Subjects were not restricted in their food consumption, but energy intake was relatively low (7930 ± 2136 kJ/day), as was vegetable intake, which may have resulted in relatively low amounts of inducer. The purpose of our food record was to estimate actual and not habitual consumption, because induction of GST enzymes occurs rapidly, in a matter of hours to days after consumption of the inducer[17, 34, 42].

Assessment of polymorphisms was reproducible, and distributions of the *GSTM1*, *GSTT1*, *GSTA1* g.-69C>T and *GSTP1* c.313A>G polymorphisms were similar to previous findings[43, 44]. The distribution of the other *GSTP1* polymorphism are not easily compared to that of the multi-ethnic population on the NCBI SNP-website (www.ncbi.nlm.nih.gov/SNP, accessed September 19th 2006).

Interindividual GST expression was highly variable in our study, which is in line with other reports[3]. Between studies, reported GST isoenzyme levels[7, 35, 45] also vary, as do GST activities[11, 12, 33, 35, 41, 46, 47]. This may be related to the method of total protein measurement, by which GST measures are normalised; these are known to recover different amounts of protein[48, 49]. It may also be related to differences in population characteristics, such as genetic differences, age, sex, smoking or dietary habits.

A general point of attention is the substrate used to measure GST activity, which was CDNB, as in most studies. The different GST enzymes contribute differently to its metabolism: the order of highest to lowest specific activity is GSTM2, GSTM1, GSTP1, GSTA, whereas GSTT1 has no activity for CDNB[1]. Total GST activity is an important measure for generic carcinogenic substrates. But, if not all GST isoenzymes or only those with lower specific activity are affected by a polymorphism or fruit and vegetable consumption, an effect may be difficult to visualize. It may, however, still be relevant, because numerous substrates are specifically metabolized by individual GST isoenzymes (e.g. N-acetoxy PhIP by GSTA1)[2, 3].

The results of the present study contribute to a better understanding of both possibilities and limitations for food mediated health effects. When investigating GST phenotype, genotype needs to be taken into account. White blood cell total GST activity may be unsuitable as a surrogate for rectal total GST activity. Thus, large and invasive studies are needed to evaluate the true effects of fruits and vegetables on rectal GSTs in human populations.

ACKNOWLEDGEMENTS

The authors wish to thank Petra Vissink and Els Siebelink for the dietary assessment; Lucy Okma, Annie van Schaik and René te Morsche for lab-related support; Jan Harryvan for *GSTM1/T1* genotyping; The endoscopy staff of the Radboud Univeristy Nijmegen Medical Center and the Canisius-Wilhelmina Hospital Nijmegen, in particular drs. Pieter Friederich and Adriaan Tan, for their support in recruitment; and all study subjects for their kind participation.

This work was supported by the Netherlands Organisation for Health Research and Development (ZonMW), grant number: 21000054, and the Dutch Digestive Diseases Foundation (MLDS), grant number WS 00-31.

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ABSTRACT

The possible interplay between cruciferous vegetable consumption, functional genetic variations in glutathione-S-transferases (GST) *M1*, *T1*, *P1* and *A1*, and colorectal adenomas, was investigated in a Dutch case-control study. The *GSTM1* and *GSTT1* deletion polymorphisms, and single nucleotide polymorphisms in *GSTP1* (A313G) and in *GSTA1* (C-69T) were assessed among 746 cases with colorectal adenomas ever in their lives and 698 endoscopy-based controls without any type of colorectal polyps. High and low cruciferous vegetable consumption was defined based on a median split in the control group. High consumption was slightly positively associated with colorectal adenomas, odds ratio (OR) 1.15, 95% confidence interval (CI) 0.92-1.44. For *GSTP1*, a positive association with higher cruciferous vegetable intake was only apparent in individuals with the low activity *GSTP1* genotype (GG genotype, OR 1.94; 95% CI 1.02-3.69). This interaction was more pronounced in men, with higher age and with higher meat intake. The *GSTA1* polymorphism may have a modifying role as well: the OR for higher intake compared to lower intake was 1.57 (0.93-2.65) for individuals homozygous for the low expression variant (TT genotype). This appeared to be stronger with younger age and higher red meat intake. Cruciferous vegetable consumption and the combined *GSTA1* and *GSTP1* genotypes showed a statistically significant interaction (p=0.034). The *GSTM1* and *GSTT1* genotypes did not seem to modify the association between cruciferous vegetable intake and colorectal adenomas. In conclusion: *GSTP1* and *GSTA1* genotypes may modulate the association between cruciferous vegetable intake and colorectal adenomas.

Chapter 3

GSTP1 and *GSTA1* polymorphisms interact with cruciferous vegetable intake in colorectal adenoma risk

Mariken J. Tijhuis
Petra A. Wark
Jac M.M.J.G. Aarts
Marleen H.P.W. Visker
Fokko M. Nagengast
Frans J. Kok
Ellen Kampman

Cancer Epidemiol Biomarkers Prev 2005;14(12):2943-51

INTRODUCTION

Diet and other lifestyle factors are thought to play a major role in the colorectal neoplastic process[1, 2]. Sporadic colorectal cancers arise from acquired DNA alterations which progressively facilitate uncontrolled cell growth, are predominantly epithelial and most are preceded by adenomas[3].

Consumption of vegetables of the family Cruciferae - in Western food patterns consisting mostly of the genus *Brassica*, including cabbage, cauliflower, Brussels sprouts and broccoli - has been associated with a decreased risk of colorectal adenomas and cancer[4], though not consistently[5-10]. Cruciferous vegetables characteristically contain glucosinolates, phytochemicals that are hydrolyzed to the biologically active isothiocyanates (ITCs)[11]. ITCs demonstrate several anti-cancer properties, including induction of phase II biotransformation enzymes such as glutathione S-transferases (GSTs, EC 2.5.1.18)[11, 12]. Thus, they may enhance the detoxification and excretion of carcinogens and prevent alterations to the DNA[13]. In addition, ITCs are substrates for GSTs[14-16] and thus GSTs contribute to the excretion of ITCs. The involvement of GSTs in ITC metabolism has led to the hypothesis that, through slower excretion of ITCs from the body in individuals with genetic variants associated with lower GST capacity, ITCs have more opportunity to exert their chemoprotective effects in these individuals[17].

Genetic polymorphisms that result in constitutively lower or absence of GST enzyme activity or expression are known for most GST isoforms[18]. Research has mostly focussed on the *GSTM1* and *GSTT1* deletion polymorphisms ("null"-genotypes). These partial gene deletions result in absence of the GSTM1 or GSTT1 enzyme, and occur among a range of 23-62% and 10-64% of the worldwide population, respectively[19]. A functional polymorphism resulting in a less active enzyme with lower thermal stability is known in the *GSTP1* gene[20]. This A to G substitution at nucleotide position 313, changing an isoleucine to a valine amino acid, is present homozygously in 4-12% of the population[21]. GSTP1 is the most abundant GST iso-enzyme in the colon[22]. A variant haplotype resulting in 3-4 fold lower expression has been identified in the *GSTA1* promoter[23], and occurs homozygously in 2-14% of the population[24].

Some epidemiologic studies indicate an interplay between crucifer consumption and metabolism by GSTs. *GSTT1*-null subjects were found to have significantly lower urinary excretion of isothiocyanates relative to *GSTT1*-positive subjects in the Singapore Chinese Health Study[25]. In this cohort study, a significantly lower risk of colon cancer was observed among individuals without both the *GSTM1* and *GSTT1* genes who had high intakes of cruciferous vegetables, compared to those with low intakes; this was not seen among individuals with one

or both genes present[26]. In a UK case-control study, a significant inverse association between high versus low cruciferous vegetable consumption and colorectal cancer risk was observed among individuals with the *GSTT1* null genotype as compared to those with one or both alleles present[27]. In the US, Lin et al. observed a lower risk of colorectal adenomas with higher consumption of cruciferous vegetables (in particular broccoli), which was statistically significant in individuals who lacked the *GSTM1* gene only[17]. The aim of the present case-control analysis was to further explore the hypothesis that in individuals with lower imputed GST capacity (here: *GSTM1* null, *GSTT1* null, *GSTP1* 313 G and *GSTA1* -69 T variants), higher cruciferous vegetable consumption is associated with a greater reduction of the risk of colorectal adenomas in a Dutch population.

METHODS

Population. Study design and population have been described before[28, 29]. Cases and controls were recruited among those undergoing endoscopy in 10 endoscopy outpatient clinics in the Netherlands between June 1997 and October 2002. Participants were informed of the study by endoscopy staff at the time of endoscopy or by mail at three-month intervals using endoscopy reports of all patients who had undergone endoscopy. All received the same information package. Eligibility criteria were: Caucasian, Dutch speaking, age 18-75 years at time of endoscopy, no hereditary colorectal cancer syndromes, no chronic inflammatory bowel disease, no history of colorectal cancer and no (partial) bowel resection. Cases had at least one histologically confirmed colorectal adenoma ever in their lives. Suitability of this case definition was confirmed by analyses restricted to cases who were first diagnosed at endoscopy of inclusion, yielding similar conclusions. Controls had no medical history of any type of polyp, confirmed by full colonoscopy (75%) or sigmoidoscopy combined with X-ray (10%). Fifteen percent of subjects did not have full visualisation of the colon, i.e. they had a sigmoidoscopy without X-ray or colonoscopy where the caecum was not reached.

The study was approved by the Medical Review Boards of all participating hospitals and of Wageningen University. The overall response rate was estimated to be 55%, varying from 35% to 90% between outpatient clinics. The total study population consisted of 1477 participants (768 cases and 709 controls). All gave their written informed consent.

Diet and other lifestyle factors. Dietary intake was assessed by a self-administered food frequency questionnaire (FFQ) developed for the Dutch European Prospective Investigation Into Cancer (EPIC) cohort and processed using the Dutch food composition table[30]. Cruciferous vegetable consumption was

calculated as the sum of raw and cooked crucifers. For cooked crucifers the frequency question was phrased 'how often do you habitually consume Brussels sprouts, cauliflower, broccoli and (other) cabbage', which covers the most commonly consumed cruciferous vegetables in the Netherlands. Frequency of cooked vegetable consumption could be indicated per day, week, month or year. If the sum of frequencies for the individual cooked vegetables was not equal to the question on total cooked vegetable consumption, the frequencies for individual vegetables were corrected proportionally[30]. Raw cabbage was measured as one of ten types of raw vegetables and frequency of consumption could be indicated always/most of the time, often, sometimes or seldom/never. Color photographs were included for 21 food items; cooked and raw crucifer portion size were estimated from a photograph containing cooked red cabbage and raw mixed salad, respectively. Frequencies and portion sizes were multiplied to obtain grams for each food item. The FFQ referred to habitual intake in the year preceding endoscopy or bowel problems. General lifestyle factors and disease-related issues, such as physical activity, smoking, self-reported family history of cancer and dietary changes due to bowel problems, were assessed by a self-administered questionnaire. Both FFQ and general questionnaire were handed at the time of endoscopy or sent within three months after endoscopy. Endoscopy-related and other medical information was abstracted from the patient's medical record.

Genotyping. EDTA treated whole blood was collected and stored at -20ºC until DNA extraction (QIAamp 96 DNA blood kit, Qiagen Inc.). An average DNA concentration of approximately 20 ng/µl was used for genotyping analyses. DNA was stored at 4ºC, in random order in 8*12 array banks. Samples were interspersed with water controls to check for cross-contamination. Laboratory staff was blinded for case-control status.

The *GSTM1* and *GSTT1* deletion polymorphisms were determined using a general multiplex polymerase chain reaction (PCR) amplification method[31]. A ß-globin gene fragment was present as a positive control. The *GSTP1* A313G polymorphism was assessed by PCR amplification followed by RFLP according to Harries[32]. The *GSTA1* C-69T polymorphism was determined by PCR-RFLP, according to Coles et al.[23]. They reported this SNP to be fully linked (in 55 liver samples) to a G to A substitution at position -52 in the promoter region[23], which has been shown to be functional in cell studies[33]. In a subsample of 93 individuals we determined the nucleotide sequence of the relevant promoter region by strand termination sequencing, and also found that the C-69T SNP and the presumed functional G–52A SNP were in 100% linkage disequilibrium. Some modifications to the Coles' PCR-RFLP protocol were made; Denaturing, annealing and elongation

times were set at 30, 30 and 45 seconds, respectively, and annealing temperature at 61°C. In the RFLP, the Eam1104I restriction enzyme was used (Fermentas Inc.). Positive controls were included in the genotyping analyses. *GSTP1* and *GSTA1* genotypes were in Hardy Weinberg Equilibrium among both controls and cases, as tested by a χ^2 test. To test reproducibility, one row out of each DNA bank (8% of the population) was genotyped in duplo; reproducibility was 100%.

Data analysis. Eighteen participants (11 cases, 7 controls) were excluded because blood was unavailable or the amount of DNA insufficient and therefore all polymorphism results were absent. Fifteen patients (11 cases, 4 controls) were excluded because of incomplete data on vegetable consumption. This resulted in a final population of 746 cases and 698 controls.

Cruciferous vegetable intakes were dichotomized by a median split based on the distribution in the control group. Conventional logistic regression was used to calculate odds ratios and 95% confidence intervals, using a single reference group (low GST capacity variant and low crucifer intake).

Evaluated as possible confounders of the relation between median split cruciferous vegetable intake and colorectal adenomas were: consumption of fruit, vegetables minus crucifers, meat; intake of energy, folate, calcium, dietary fibre, vitamin C, coffee, alcohol, and saturated fat; physical activity, use of NSAIDs, smoking, education, hormone replacement therapy, body mass index, abdominal problems, defecation problems and self-reported family history of colorectal cancer. Folate intake, age (four categories) and sex changed the β-estimate by 35%, 20% and 20%, respectively, and were included in the model. Energy intake did not affect the estimates, but was included for comparability with other studies.

The p-value for interaction was calculated by χ^2 test comparing the –2 log likelihood (-2LL) values of the models with and without crucifer-by-genotype interaction term(s). P-values lower than 0.05 were considered statistically significant. Genotypes were considered individually and in combinations; grouping rationales were: correspondence with the literature (*GSTM1*/*T1*), high colonic gene expression (*GSTP1*/*T1*), high hepatic gene expression (*GSTA1*/*M1*), and posterior combination of most promising separate genotypes (*GSTP1*/*A1*).

The crucifer-by-genotype interaction was further studied in strata of age (higher and lower than 55 years), sex, smoking (ever and never) and meat consumption (fresh red and processed, higher and lower than median intake). Stratifying rationales were: indication from the literature for a possible modification by age[34] and sex[35], and our hypothesis that individuals with high exposure to (pro)carcinogens from meat and cigarettes may benefit most from low activity GST variants in combination with high cruciferous vegetable intake. For

comparison, the cruciferous vegetable median split based on population controls was maintained. Sex-specific median cut-off points yielded similar estimates as population-based medians, to enable comparison we present the latter.

Statistical analyses were performed using SAS software, version 9.1 (SAS Institute, Cary, NC).

RESULTS

Table 1 shows the characteristics of the study population. The control group included more women than the case group. Cases were older and more often smokers. Controls more often had defecation problems or other abdominal complaints than cases. Cases, on the other hand, more often experienced rectal bleeding and more often had a history of endoscopy and came for surveillance. Cases consumed more cruciferous vegetables and total vegetables than controls, and had a higher alcohol intake. The distribution of GST variants did not differ between cases and controls.

In table 2, the associations between cruciferous vegetable intake, GST polymorphisms and colorectal adenomas are presented. Consumption of cruciferous vegetables was slightly positively associated with colorectal adenomas. A positive association with higher cruciferous vegetable intake was more obvious for the *GSTP1* low activity genotype (GG) and *GSTA1* low expression genotype genotype (TT). The combination of *GSTP1* and *GSTA1* genotypes showed a statistically significant interaction with cruciferous vegetable consumption; adenoma risk was higher with high cruciferous vegetable intake for subjects with one or both of the low capacity genotypes (*GSTP1* GG or *GSTA1* TT), but not for subjects with both high capacity variants (*GSTP1* AA and *GSTA1* CC). In the low crucifer group, the high capacity variants appeared to have a higher adenoma risk than the low capacity variants. *GSTM1* and *GSTT1* deletion polymorphisms did not seem to affect the association between cruciferous vegetable intake and colorectal adenomas.

When, instead of a median split, we used tertiles of cruciferous vegetable intake, the high crucifer intake*low *GSTP1* activity (GG) variant and high crucifer intake*low *GSTA1* expression (TT) variant still had the highest adenoma risk: OR 1.52 (95% CI 0.70-3.28, p value for interaction 0.081), and 2.27 (1.21-4.24, p=0.054), respectively. Cruciferous vegetable consumption expressed as a continuous variable showed significant interaction with the *GSTA1* variants (p=0.030).

Table 1: Population characteristics

Characteristic		Cases n=746	Controls n=698
General			
Sex	% female	46.5	61.8
Age (mean±sd)	years	59.0±10.1	51.5±13.6
Smoking status	% ever	66.8	55.3
BMI (mean±sd)		26.1±3.9	25.5±4.1
Physical activity	% low	38.2	33.2
Regular NSAID use (≥12/yr)	% yes	26.7	29.7
Education	% low[a]	35.9	33.0
Medical, % yes			
Family history of colorectal cancer		23.2	20.0
Abdominal complaints		34.0	59.0
Defecation problems		25.7	43.4
Blood loss per anum		30.6	18.9
Surveillance/follow-up		48.0	6.1
Dietary intake, median (10-90 percentile)			
Energy	kJ/d	8394 (5889-11800)	8113 (5452-11808)
Cruciferous	g/wk	137 (41-322)	129 (32-289)
vegetables	freq/wk	0.9 (0.3-1.9)	0.8 (0.2-1.7)
	g/d	114 (69-180)	109 (68-169)
Total vegetables[b]	freq/wk	7.0 (4.6-11.5)	7.0 (4.7-11.0)
Total fruit	g/d	127 (18-367)	125 (27-350)
	freq/wk	7.0 (1.0-21.0)	7.0 (1.5-21.0)
Folate	µg/d	194 (139-272)	185 (131-260)
Dietary fibre	g/d	23 (15-32)	23 (15-32)
	g/d	107 (40 – 177)	102 (35 – 172)
Total meat	freq/wk	14.5 (5.9-31.0)	14.0 (5.4-29.9)
Fresh red meat	g/d	60 (16 – 100)	53 (13 – 96)
	freq/wk	4.3 (1.7-6.0)	4.2 (1.6-6.1)
Processed meat	g/d	30 (6 – 75)	29 (5 – 73)
	freq/wk	9.2 (1.03-25.4)	8.4 (1.09-24.1)
Alcohol	g/day	9 (0-42)	4 (0-31)
Coffee	cups/day	4 (2-7)	4 (0-8)
Genotype distribution, %			
GSTM1	present/null	48.6/51.4	45.9/54.1
GSTT1	present/null	82.0/18.0	81.8/18.2
GSTP1	AA/AG/GG	42.5/45.3/12.2	42.8/45.3/11.9
GSTA1	CC/CT/TT	33.8/47.9/18.3	34.8/47.1/18.1

[a]primary school or lower vocational training only

[b]definition of total vegetables includes non-starch legumes (string beans and peas) and excludes potato and vegetable juice

Table 2: Interplay between cruciferous vegetable intake (g/wk), GST genotypes, and the risk of colorectal adenomas

	n (cases/controls)		Age + sex adjusted OR (95% CI) *p for interaction*[a]		Multiple adjusted* OR (95% CI) *p for interaction*[a]	
Crucifer intake[b]	≤ 129 g/wk	> 129 g/wk	≤ 129 g/wk	> 129 g/wk	≤ 129 g/wk	> 129 g/wk
All genotypes	336/349	410/349	1	1.22 (0.98-1.52)	1	1.15 (0.92-1.44)
Individual genotypes[c]						
GSTM1						
null	168/185	213/192	1	1.18 (0.87-1.59)	1	1.12 (0.83-1.53)
present	166/163	194/157	1.09 (0.79-1.49)	1.39 (1.01-1.89)	1.09 (0.80-1.50)	1.30 (0.94-1.79)
			0.72		*0.81*	
GSTT1						
null	62/61	71/66	1	1.13 (0.68-1.88)	1	1.08 (0.64-1.80)
present	272/287	336/283	0.97 (0.64-1.46)	1.20 (0.80-1.80)	0.99 (0.65-1.49)	1.15 (0.76-1.73)
			0.75		*0.79*	
GSTP1						
[d]GG	34/43	56/40	1	2.06 (1.09-3.89)	1	1.94 (1.02-3.69)
AG	151/169	183/147	1.22 (0.72-2.07)	1.61 (0.95-2.71)	1.22 (0.72-2.06)	1.50 (0.89-2.55)
AA	148/137	166/162	1.43 (0.84-2.43)	1.39 (0.82-2.34)	1.40 (0.82-2.39)	1.30 (0.76-2.20)
			0.10		*0.11*	
GSTA1						
[d]TT	53/63	82/63	1	1.67 (1.00-2.81)	1	1.57 (0.93-2.65)
CT	166/169	187/160	1.26 (0.81-1.97)	1.42 (0.91-2.21)	1.24 (0.79-1.95)	1.32 (0.84-2.06)
CC	113/117	136/126	1.19 (0.75-1.91)	1.39 (0.87-2.21)	1.17 (0.73-1.88)	1.30 (0.82-2.08)
			0.42		*0.43*[e]	
Genotype combinations						
GSTM1/T1						
both null	30/33	32/36	1	1.15 (0.56-2.35)	1	1.11 (0.54-2.29)
M1 or T1 null	170/180	220/186	1.18 (0.67-2.07)	1.37 (0.79-2.39)	1.21 (0.69-2.12)	1.33 (0.76-2.32)
both present	134/135	155/127	1.16 (0.66-2.06)	1.53 (0.86-2.71)	1.20 (0.67-2.11)	1.46 (0.82-2.59)
			0.86		*0.91*	
GSTP1/A1						
P1 GG and/or A1 TT	81/100	129/94	1	1.87 (1.23-2.84)	1	1.76 (1.16-2.69)
remaining genotypes	196/211	216/196	1.23 (0.85-1.77)	1.37 (0.95-1.99)	1.22 (0.84-1.77)	1.28 (0.88-1.87)
P1 AA and A1 CC	54/38	60/59	1.71 (1.00-2.92)	1.33 (0.81-2.16)	1.69 (0.99-2.88)	1.24 (0.76-2.03)
			0.033		*0.034*	
GSTA1/M1						
A1 CT/TT and M1 null	118/127	143/125	1	1.18 (0.82-1.70)	1	1.12 (0.78-1.62)
A1 CC and M1 null	49/58	66/67	0.93 (0.58-1.50)	1.05 (0.67-1.64)	0.94 (0.58-1.51)	1.03 (0.66-1.60)
A1 CT/TT and M1 pres	100/104	124/98	1.03 (0.70-1.52)	1.38 (0.94-2.02)	1.04 (0.70-1.54)	1.30 (0.88-1.91)
A1CC and M1 pres	64/59	69/59	1.11 (0.70-1.74)	1.33 (0.85-2.09)	1.10 (0.70-1.74)	1.25 (0.79-1.97)
			0.96		*0.98*	
GSTP1/T1						
P1 AG/GG and T1 null	35/35	45/34	1	1.36 (0.69-2.68)	1	1.26 (0.64-2.49)
P1 AA and T1 null	27/26	26/32	1.03 (0.49-2.18)	0.91 (0.44-1.89)	0.99 (0.46-2.09)	0.87 (0.41-1.81)
P1 AG/GG and T1 pres	149/176	194/153	0.88 (0.51-1.52)	1.30 (0.76-2.22)	0.89 (0.51-1.53)	1.23 (0.71-2.11)
P1 AA and T1 pres	120/111	137/130	1.10 (0.63-1.93)	1.08 (0.62-1.88)	1.09 (0.62-1.92)	1.01 (0.58-1.76)
			0.34		*0.37*	

*adjusted for age (in 4 categories: <45, <55, <65, <75 y), sex, energy intake (kJ/d) and folate intake (g/d)

[a]calculated as the LR test comparing the models with and without crucifer-by-genotype interaction terms

[b]mean cruciferous vegetable intake was 73.1±36.3 g/wk and 70.3±36.6 g/wk for cases and controls, respectively, in the lower-than-median intake group; and 242.5±108.4 g/wk and 230.3±109.2 g/wk, respectively, in the higher-than-median intake group

[c]numbers do not always add up to numbers under 'all genotypes' due to missing genotype-data (M1,T1: 6; P1:8; A1:9)

[d]least active/expressed variant(s)

[e]when cruciferous vegetable intake is considered as a continuous variable, crucifer*genotype interaction is significant, p=0.030

About half (n=394) of all cases did not have a history of colorectal polyps. Analyses restricted to these newly diagnosed cases yielded similar conclusions, with more pronounced results for *GSTP1* and *GSTA1* genotypes; for *GSTP1*, the OR for high crucifer intake**GSTP1* GG-genotype was 2.90 (95% CI 1.32-6.39; p value for interaction 0.029); for *GSTA1*, the OR for high crucifer intake**GSTA1* TT-genotype was 1.92 (1.03-3.58, p= 0.17); and for the combined *GSTA1* and *GSTP1* genotypes, the p value for interaction was 0.0023, with an OR of 2.31 (1.40-3.84) for the high intake*low GST capacity combination. Median intake of cruciferous vegetables among incident cases was slightly, but not statistically significantly, lower than intake among prevalent cases (143 vs.135 g/week, p=0.48).

In fifteen percent of our controls a full visualisation of the colon was not accomplished. However, restriction to controls with full visualisation (n=591) yielded similar results as analyses including all controls (data not shown).

Further evaluation in strata of age and sex showed differences in colorectal adenoma risk (table 3). *GSTP1* genotypes and cruciferous vegetable intake showed a statistically significant interaction in the higher, but not in the lower age category; The *GSTA1* genotype showed a near-significant interaction with cruciferous vegetable medians in the lower age category, only. In men, but not in women, there was statistically significant interaction between the *GSTP1* genotype and cruciferous vegetable intake. Noteworthy in this respect is the lower prevalence of the *GSTP1* GG genotype in men: 10.2% (cases 10.6%, controls 9.7%) versus 13.6% for women (cases 14.0%, controls 13.3%) (p=0.054). The overall colorectal adenoma risk (without genotype stratification) with higher cruciferous vegetable consumption appeared higher in men. In individuals with a positive family history of colorectal cancer, prevalence of the *GSTA1* low expression genotype (*GSTA1* TT) was significantly lower than in individuals without a positive family history of cancer (13.5% vs. 19.1%, p=0.027). The overall colorectal adenoma risk with higher cruciferous vegetable consumption was higher in individuals with a positive family history: OR 1.83 (1.12-3.01). Unfortunately, power was too low for gene-environment analyses in separate strata of family history.

In strata of possible environmental exposure to carcinogens (table 4), the higher risk with higher cruciferous vegetable intake associated with the low activity *GSTP1* variant appeared more pronounced in subgroups of higher (red and processed) meat consumption and slightly in non-smokers. For *GSTA1*, results appeared more pronounced in the higher red meat subgroup.

Table 3: Interplay between cruciferous vegetable intake (g/wk), *GSTP1* and *GSTA1* genotype, and the risk of colorectal adenomas in subgroups of age and sex

		n (cases/controls)		OR (95% CI)* *p for interaction*[a]		n (cases/controls)		OR (95% CI)* *p for interaction*[a]	
Crucifer intake[b]		≤ 129 g/wk	> 129 g/wk	≤ 129 g/wk	> 129 g/wk	≤ 129 g/wk	> 129 g/wk	≤ 129 g/wk	> 129 g/wk
AGE		≤ 55 years[c]				> 55years[c]			
All genotypes		119/205	134/195	1	1.14 (0.82-1.59)	217/144	276/154	1	1.17 (0.86-1.58)
GSTP1	[d]GG	11/21	15/27	1	1.10 (0.41-2.94)	23/22	41/13	1	3.22 (1.35-7.69)
	AG	51/104	59/76	0.93 (0.41-2.09)	1.38 (0.61-3.15)	100/65	124/71	1.50 (0.76-2.93)	1.61 (0.83-3.14)
	AA	55/80	57/92	1.26 (0.56-2.86)	1.13 (0.50-2.56)	93/57	109/70	1.54 (0.78-3.05)	1.45 (0.74-2.84)
				0.34				*0.040*	
GSTA1	[d]TT	14/39	27/35	1	2.31 (1.03-5.18)	39/24	55/28	1	1.22 (0.61-2.45)
	CT	63/95	55/91	2.00 (0.99-4.04)	1.58 (0.77-3.22)	103/74	132/69	0.90 (0.50-1.65)	1.19 (0.65-2.17)
	CC	41/71	49/69	1.58 (0.76-3.29)	2.12 (1.02-4.40)	72/46	87/57	0.98 (0.52-1.85)	0.95 (0.51-1.77)
				0.060				*0.67*	
SEX		Male				Female			
All genotypes		190/145	209/122	1	1.24 (0.89-1.72)	146/204	201/227	1	1.05 (0.77-1.44)
GSTP1	[d]GG	18/19	24/7	1	3.75 (1.25-11.2)	16/24	32/33	1	1.33 (0.57-3.08)
	AG	80/73	101/54	1.25 (0.59-2.62)	1.99 (0.94-4.22)	71/96	82/93	1.16 (0.55-2.44)	1.14 (0.54-2.39)
	AA	91/53	82/61	1.93 (0.91-4.09)	1.45 (0.68-3.06)	57/84	84/101	1.04 (0.49-2.22)	1.12 (0.54-2.35)
				0.0095				*0.82*	
GSTA1	[d]TT	35/29	38/21	1	1.37 (0.65-2.92)	18/34	44/42	1	1.72 (0.81-3.64)
	CT	83/63	103/61	1.06 (0.57-1.95)	1.38 (0.75-2.54)	83/106	84/99	1.49 (0.76-2.90)	1.29 (0.66-2.54)
	CC	70/53	66/40	1.16 (0.62-2.18)	1.25 (0.65-2.41)	43/64	70/86	1.19 (0.58-2.45)	1.35 (0.68-2.70)
				0.83				*0.28*	

*adjusted for age (in 4 categories: <45, <55, <65, <75 y), sex, energy intake (kJ/d) and folate intake (g/d), if applicable

[a]calculated as the LR test comparing the models with and without crucifer-by-genotype interaction terms

[b]mean cruciferous vegetable intake was 73.1±36.3 g/wk and 70.3±36.6 g/wk for cases and controls, respectively, in the lower-than-median intake group; and 242.5±108.4 g/wk and 230.3±109.2 g/wk, respectively, in the higher-than-median intake group

[c]age in the lower and higher age category was 44.1±8.8 years and 64.7±5.6 years, respectively

[d]least active/expressed variant(s)

Table 4: Interplay between cruciferous vegetable intake (g/wk), *GSTP1* and *GSTA1* genotype, and the risk of colorectal adenomas in subgroups of meat consumption and smoking

		n (cases/controls)		OR (95% CI)*		n (cases/controls)		OR (95% CI)*	
				p for interaction[a]				*p for interaction*[a]	
Crucifer intake[b]		≤ 129 g/wk	> 129 g/wk	≤ 129 g/wk	> 129 g/wk	≤ 129 g/wk	> 129 g/wk	≤ 129 g/wk	> 129 g/wk
RED MEAT[c]				≤53 g/d				> 53 g/d	
All genotypes		161/173	161/176	1	0.93 (0.66-1.30)	175/176	249/173	1	1.36 (1.00-1.86)
GSTP1	[d]GG	17/19	26/24	1	1.44 (0.58-3.61)	17/24	30/16	1	2.67 (1.07-6.69)
	AG	65/85	68/77	1.05 (0.48-2.28)	0.97 (0.44-2.11)	86/84	115/70	1.39 (0.67-2.89)	2.04 (0.98-4.22)
	AA	78/69	65/75	1.33 (0.61-2.89)	1.08 (0.49-2.38)	70/68	101/87	1.34 (0.63-2.82)	1.48 (0.72-3.04)
				0.56				*0.22*	
GSTA1	[d]TT	29/30	28/33	1	1.05 (0.48-2.28)	24/33	54/30	1	2.36 (1.14-4.87)
	CT	86/87	64/76	1.12 (0.59-2.12)	0.78 (0.40-1.51)	80/82	123/84	1.41 (0.74-2.67)	2.01 (1.07-3.77)
	CC	46/56	67/67	0.85 (0.43-1.70)	1.04 (0.53-2.02)	67/61	69/59	1.66 (0.86-3.22)	1.66 (0.86-3.24)
				0.30				*0.16*	
PROCESSED MEAT[e]				≤ 29 g/d				> 29 g/d	
All genotypes		156/160	189/189	1	0.96 (0.69-1.33)	180/189	221/160	1	137 (1.00-1.88)
GSTP1	[d]GG	19/18	28/24	1	1.23 (0.50-3.03)	15/25	28/16	1	3.25 (1.27-8.30)
	AG	73/68	79/89	1.11 (0.51-2.40)	0.81 (0.38-1.75)	78/101	104/58	1.42 (0.68-2.98)	2.87 (1.35-6.10)
	AA	62/74	79/76	0.87 (0.40-1.89)	1.03 (0.48-2.22)	86/63	87/86	2.24 (1.05-4.75)	1.66 (0.79-3.49)
				0.32				*0.0016*	
GSTA1	[d]TT	23/33	42/36	1	1.85 (0.88-3.89)	30/30	40/27	1	1.41 (0.67-2.98)
	CT	82/79	81/87	1.69 (0.88-3.25)	1.30 (0.68-2.51)	84/89	106/74	0.94 (0.50-1.75)	1.36 (0.72-2.54)
	CC	48/48	63/66	1.47 (0.72-2.97)	1.38 (0.70-2.72)	65/69	73/60	1.00 (0.53-1.91)	1.27 (0.66-2.43)
				0.14				*0.92*	
SMOKING			Never				Ever		
All genotypes		102/146	146/165	1	1.19 (0.82-1.73)	234/202	264/184	1	1.16 (0.87-1.55)
GSTP1	[d]GG	12/18	24/15	1	3.32 (1.16-9.53)	22/25	32/25	1	1.44 (0.64-3.26)
	AG	48/76	54/72	1.14 (0.48-2.71)	1.17 (0.49-2.78)	103/93	129/75	1.28 (0.65-2.50)	1.79 (0.91-3.50)
	AA	40/53	66/78	1.27 (0.52-3.09)	1.34 (0.57-3.15)	108/84	100/84	1.46 (0.74-2.85)	1.30 (0.66-2.57)
				0.12[f]				*0.29*	
GSTA1	[d]TT	14/24	31/30	1	2.16 (0.88-5.30)	39/39	51/33	1	1.37 (0.71-2.64)
	CT	57/71	65/80	1.45 (0.64-3.29)	1.25 (0.56-2.75)	109/98	122/80	1.16 (0.67-2.01)	1.45 (0.81-2.53)
	CC	29/52	48/55	0.96 (0.41-2.27)	1.45 (0.64-3.32)	84/65	88/71	1.32 (0.74-2.35)	1.26 (0.71-2.23)
				0.14				*0.59*	

*adjusted for age (in 4 categories: <45, <55, <65, <75), sex, energy intake and folate intake

[a]calculated as the LR test comparing the models with and without crucifer-by-genotype interaction terms

[b]mean cruciferous vegetable intake was 73.1±36.3 g/wk and 70.3±36.6 g/wk for cases and controls, respectively, in the lower-than-median intake group; and 242.5±108.4 g/wk and 230.3±109.2 g/wk, respectively, in the higher-than-median intake group

[c]fresh red meat consumption defined as sum of pork, beef and unclassified (non-organ) meats; consumption in the lower and higher category was 28.7±15.2 g/d and 82.1±21.2 g/d,respectively

[d]least active/expressed variant(s)

[f]p-value Lemeshow goodness-of-fit test: 0.049

[e]processed meat defined as preserved and ready-to-eat meat products; consumption in the lower and higher category was 13.7±8.2 g/d and 57.8±32.7 g/d, respectively

DISCUSSION

In this endoscopy-based case-control study of colorectal adenomas, the *GSTP1* A313G and *GSTA1* C-69T single nucleotide polymorphisms appear to modify the association between cruciferous vegetable consumption and colorectal adenoma risk, though with results that contradict our hypothesis that low capacity GST genotypes benefit (most) from higher consumption: compared with the low crucifer consumption*low GST capacity variant combination, the highest adenoma risk was observed in the high crucifer consumption*low GST capacity variant combination.

There was no indication for an interplay between cruciferous vegetable consumption and *GSTM1* or *GSTT1* deletion polymorphisms in this population.

Our study has strengths and weaknesses. The response rate was about 55%, and varied rather widely by clinic, depending on local recruitment factors. However, selection procedures were identical for cases and controls, reducing the possibility for differential selection bias. Unfortunately we do not have sufficient data on patients not participating in the study to further evaluate the possible selection bias due to non-response. Our control group underwent endoscopy, mostly because of bowel problems, and as such may not be fully representative of the average individual without adenomas in the population. Therefore, risk estimates cannot be extrapolated to the general population inadvertently. However, adenomas are fairly prevalent in the population and often do not give symptoms[36]. Thus, the fact that our control status was defined by endoscopy can be seen as an advantage. Full visualisation of the colon was achieved for most controls and restriction of analyses to this group did not change results. Combined with the fact that adenomas were histologically confirmed, case-control misclassification is unlikely. Bowel complaints may lead to change in food habits. Only few participants, however, indicated an increase in vegetable intake (16 cases, 18 controls) or a decrease in cabbage intake (8 cases, 4 controls) specifically. Exclusion of these participants from analyses did not change our conclusion. Excluding those with bowel problems weakened results for *GSTP1*, strengthened results for *GSTA1* and yielded similar results for the *GSTP1/A1* combination, though statistical significance was lost due to small numbers.

Our case group consisted of new as well as previously diagnosed cases. Restriction of analyses to new cases did not change our conclusions and thus information bias due to differences in recall of dietary habits and/or time span of adenoma diagnosis and related surveillance is likely to have been limited. Information on reproducibility and validity of our cruciferous vegetable measurement is not available and we cannot rule out some degree of exposure

misclassification. For total vegetables, the reproducibility was 0.76 and 0.65 for men and women, respectively, and relative validity was 0.38 and 0.31 for men and women, respectively, which is in the range of estimates that others find for FFQs[30]. Genotype misclassification is assumed to be low, as measurement and scoring was blind, negative and positive controls were included and duplos were assessed with excellent reproducibility. The genotypes are sufficiently prevalent and the study population sufficiently large to render power adequate for most of the analyses.

Concluding: our study has many strengths, yet risk of selection and information bias remains a point of attention in case-control studies. Bias cannot be ruled out in the explanation of our finding of a slightly higher adenoma risk with higher cruciferous vegetable consumption. However, we add to an existing literature, of both case-control and cohort studies, in which increased risks, significant and non-significant, of colorectal adenomas or cancer with higher intakes of cruciferous vegetables have been reported[5-8, 10]. Unlike these studies, we also incorporated genetic polymorphisms in our design.

We hypothesized that inherited decreased GST-capacity, which presumably results in a longer biological half-life of phytochemicals from cruciferous vegetables, may confer increased chemopreventive potential when these vegetables are consumed at higher levels. Other studies preceded ours: for the *GSTM1* and/or *GSTT1* deletion genotypes, lower colorectal adenoma[17] and cancer[26, 27, 34] risks were observed with higher cruciferous vegetable intake. In the case-control study by Lin et al., however, the observed decreased risk of colorectal adenomas with higher cruciferous vegetable intake in *GSTM1* null subjects did not apply to all types of crucifers (i.e. not to cabbage, cauliflower or Brussels sprouts), but was restricted to broccoli[17]. We were unable to separate crucifer type, but we generated toplists of cruciferous vegetables in the 1998 Dutch National Food Consumption Survey[37] and found that broccoli contributed only about 8% to the total amount of crucifers by weight. The cruciferous vegetable contributing most in this population was cauliflower (about 30%). Interestingly, in some of the aforementioned other studies reporting increased risk, cauliflower and cabbage contributed 97% to cruciferous vegetable intake[8], or the reported risk was for cauliflower and cabbage specifically[5, 7]. Cruciferous vegetables differ in their composition of glucosinolates[38] and different (patterns of) consumption may be one reason why we were unable to reproduce the findings by Lin et al. in our population[17]. Lin et al. also investigated the *GSTT1* genotype[39], but without convincing results, whereas the interplay between cruciferous vegetables and GST genotypes reported by Turner et al. was limited to *GSTT1* and could not be seen for *GSTM1*[27]. The differential ITC excretion levels among individuals with different

GSTT1 genotypes found by Seow[25] was not replicated by Fowke et al. and both found urinary ITC levels unaffected by *GSTM1* genotype[25, 40]. The use of genotype assays that are able to differentiate between the presence of one or two alleles[41] may help in creating a more consistent view concerning the modifying effects by *GSTM1* and *GSTT1* genotypes.

For the *GSTP1* A313G polymorphism, Fowke et al. found a significant positive trend between cruciferous vegetable intake and fasting first-morning urinary ITC excretion in the high activity *GSTP1* AA genotype[40]. No association between urinary excretion of total ITC and the *GSTP1* A313G genotype was observed by Seow et al.[25], however, nor did they find an interplay between *GSTP1* genotype, high cruciferous vegetable intake and colorectal cancer among Singapore Chinese[26]. It must be noted that the frequency of the *GSTP1* 313G allele among Asian subjects is low (3.5-5%), resulting in low power, and this may explain lack of association. The results of our study do not support the hypothesis that imputed decreased GSTP1 or GSTA capacity in combination with high crucifer intake protects against colorectal adenoma, but they may support an interaction between cruciferous vegetable consumption and these genotypes. Both the *GSTP1* low activity variant (GG genotype) and the *GSTA1* low expression variant (TT genotype) appeared to increase the risk of colorectal adenomas with higher cruciferous vegetable intake. This higher adenoma risk with a low capacity GST variant could reflect directly the slower processing of genotoxic compounds associated with cruciferous vegetable intake. Genotoxicity of glucosinolate breakdown products is supported by bacterial and cell assays[42, 43]. In the human situation, however, it is believed that a variety of mechanisms operate to prevent the genotoxic effects observed *in vitro* and that toxicity may therefore occur only at exposure doses exceeding human intake 100-fold[44]. However, this does suggest there is a certain dependency on a well functioning biotransformation system. An indirect possibility for a higher adenoma risk, that finds some support in human studies, is the prolonged stimulation of phase I enzymes, notably *CYP1A2*, by glucosinolate-derived (indole) ITCs that are metabolized by GSTs[45-47]. If this is not accompanied by sufficient conjugating activity, this might result in a net increased metabolic activation of (pro)carcinogens. In individuals carrying the two most active variants of the *GSTA1* and *GSTP1* gene homozygously, there is no indication of higher adenoma risk with higher crucifer intake, possibly suggesting that sufficient detoxification is provided by these high capacity variants. There does appear to be a genotype effect in the low cruciferous vegetable group.

GSTs have overlapping substrate specificities[18], and the iso-forms may interact. Ketterer and coworkers suggested a link between GSTA and GSTM1: GSTA

sequesters ITC-conjugates and converts them slowly back to the active ITC[15, 48], but only in the *GSTM1* null individual; in the *GSTM1*-positive individual, excretion is favored. It is possible then, that a higher availability of GSTA, as partly determined by *GSTA1* C-69T genotype, might result in a higher amount of conservation, especially in *GSTM1* null individuals. We found no evidence for an interplay between the *GSTM1* and *GSTA1* genotypes in our study. GSTM1 and GSTA are expressed at a low level in the colon[22], but are highly expressed in the liver[49]. They are relevant for the colon because glucosinolate metabolites are also delivered to the colonic crypts via the colonic blood supply, after absorption from the small intestine and passage through the liver[50]. The combined genotypes of the genes most expressed in the colon, i.e. *GSTP1* and *GSTT1*, also did not show a clear effect on the association between colorectal adenomas and crucifer intake.

GSTs have dual roles in relation to glucosinolates: apart from their metabolizing roles, a number of GSTs, e.g. *GSTP1* and *GSTA1*, appear to be inducible by glucosinolate breakdown products[35, 51]. Although in the *GSTA1* gene no electrophile responsive element or xenobiotic responsive element has been found, other transcription factor binding sites have been identified. The differential expression of the *GSTA1* genotypes defined by the C-69T substitution has been attributed to alteration of the binding of the ubiquitously expressed transcription factor Sp1[33]. The glucosinolate-derived indole ITC indol-3-carbinol (I3C) may influence this binding, demonstrating the complicated nature of the GST-glucosinolate relation[52].

Our subgroup analyses showed differences in subgroups of age, sex, meat consumption and smoking, which we may not be able to fully disentangle from one another. They may be due to chance. Glutathione availability decreasing with age may play a role[53]. Generation and sex may influence dietary habits. Also, GST responses to cruciferous vegetable diets may differ between men and women[35]. N-acetoxy PhIP, one of the most important (pro)carcinogens in cooked meat, is metabolized by GSTα and to a lesser extent by GSTP1[54] and thus subjects with low capacity variants may be less equipped to detoxify[55, 56]. If slower processing of glucosinolate-derived compounds leads to prolonged phase I activation, then subjects with low capacity GST variants and higher exposure to (meat) carcinogens may be burdened even more by these carcinogens. Similarly, GSTP1 is involved in the detoxification of polycyclic aromatic hydrocarbons (PAHs) present in cigarette smoke and a potential by-product of meat processing. Slattery et al. also reported a modifying role for smoking and suggested smoking may influence the balance of phase II and phase I biotransformation enzymes[4], but how this applies to our results is not clear.

We conclude that genetic variation in the *GSTP1* and the *GSTA1* gene may modulate the relation between cruciferous vegetables and colorectal adenomas. In this population, the advantage of hypothetical prolonged stimulation of phase II enzymes may not outweigh the disadvantage of lower GST enzyme capacity. Further research may explore the specific effects of the different cruciferous vegetable types. Phenotyping of phase I enzymes (e.g. *CYPs*) in relation to phase II GST polymorphisms or proteins may shed more light on the balance between phase I bioactivation and phase II detoxification of (pro)carcinogens. Also, genetic variation in GST inducibility (i.e. in regulatory sequences) may be taken into account.

ACKNOWLEDGEMENTS

We thank Dr. Brian Coles for kindly supplying positive controls for the GSTA1 genotypes.

We thank Edine Tiemersma, Elly Monster, Maria van Vugt, Dorien Voskuil, Brenda Diergaarde and Maureen van den Donk for their roles in the conduct of the case-control study, as well as Annelies Bunschoten and Jan Harryvan for their support in the genotype assessment, Bram-Sieben Rosema for datacleaning and preliminary analyses, Marga Ocké from the National Institute of Public Health and the Environment for the dietary calculations, and Saskia Meyboom for generating the cruciferous vegetable toplists in the Dutch National Food Consumption Survey. We also thank all persons who participated in the study and the endoscopy staff of the following Dutch hospitals where the participants were recruited: Slingeland Ziekenhuis (Doetinchem), Ziekenhuis Gelderse Vallei (Ede), Radboud University Nijmegen Medical Centre (Nijmegen), Antonius Ziekenhuis (Nieuwegein), Meander Medisch Centrum (Amersfoort), Ziekenhuis Rijnstate (Arnhem), Ziekenhuis Rivierenland (Tiel), Slotervaart Ziekenhuis (Amsterdam), Jeroen Bosch ziekenhuis (Den Bosch), and Canisius Wilhelmina Ziekenhuis (Nijmegen).

This work was supported by the Netherlands Organisation for Health Research and Development (ZonMW), grant number: 21000054.

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Abstract

NAD(P)H:quinone oxidoreductase (NQO1) is an inducible detoxification enzyme relevant for colorectal cancer biochemoprevention. We evaluated the influence of recent fruit and vegetable (F&V) consumption and polymorphisms in NQO1 and transcription factor NFE2L2 on rectal NQO1 phenotype and also whether white blood cell (WBC) NQO1 activity reflects rectal activity. Among 94 sigmoidoscopy-patients, we assessed F&V consumption by dietary record and determined the *NQO1* c.609C>T and g.-718A>G and *NFE2L2* g.-650C>A, g.-684G>A and g.-686A>G polymorphisms. *NQO1* mRNA level was measured in rectal biopsies, NQO1 activity in rectal biopsies and WBC. Consumption of F&V did not yield higher mRNA level or activity, but rather appeared to have a repressive effect. Rectal activity was higher among *NQO1* 609CC-genotypes as compared to 609CT-genotypes ($p<0.0001$; 609TT-genotypes were absent), whereas mRNA was higher among 609CT-genotypes ($p<0.001$). mRNA and activity correlated among *NQO1* 609CC-genotypes ($r=0.50$, $p=0.0001$) but not among 609CT-genotypes ($r=0.14$, $p=0.45$). The *NFE2L2* -684A-allele was associated with higher mRNA levels ($p=<0.05$). The other polymorphisms did not affect phenotype significantly. WBC and rectal activity did not correlate. In conclusion, genetic variation, especially the *NQO1* 609C>T polymorphism, is a more important predictor of rectal NQO1 phenotype than F&V consumption. WBC NQO1 activity is not a good surrogate for rectal activity.

Chapter 4

The influence of fruit and vegetable consumption and genetic variation on NAD(P)H:quinone oxidoreductase (NQO1) phenotype in an endoscopy-based population

Mariken J. Tijhuis
Anne-Marie J.F. Boerboom
Marleen H.P.W. Visker
Liesbeth Op den Camp
Fokko M. Nagengast
Adriaan C.I.T.L. Tan
Ivonne M.C.M. Rietjens
Frans J. Kok
Jac M.M.J.G. Aarts
Ellen Kampman

Nutrition and Cancer, in press

INTRODUCTION

NAD(P)H:quinone oxidoreductase (NQO1; EC 1.6.5.2) is involved in the 2-electron reductive biotransformation of quinones, which are highly reactive molecules with carcinogenic potential[1]. Quinones have important roles in cellular respiration and are ubiquitous in nature[2]. Epithelial tissues, such as of the digestive tract, produce relatively high levels of NQO1 protein, which is thus readily available to act on quinones entering the body[3, 4]. NQO1 detoxifies or activates the toxic action of a quinone depending on the stability of the hydroquinone that is formed[1]. Despite its contrasting potential, increasing the NQO1 capacity is expected to overall inhibit carcinogenesis. Besides facilitating the excretion of exogenous quinones, a role for NQO1 has been proposed in antioxidant protection through reduction of endogenous quinones and prevention of one-electron redox cycling thus inhibiting formation of DNA-damaging reactive oxygen species[5], and in stabilization of the major tumor suppressor gene p53[5]. Sufficient NQO1 capacity, both constitutive and induced, is therefore important for protection against quinone-type carcinogens and oxidative stress.

The human NQO1 gene is inducible[6-8]. An electrophile-responsive element (EpRE), wich mediates the regulation of many detoxifying enzymes, has been identified in its 5'-flanking region[9]. EpRE mediated upregulation of protective biotransformation enzymes, by plant food components such as isothiocyanates, flavonoids, carotenoids, sulfides and phenols, can contribute importantly to prevention of carcinogenesis[10, 11]. This may be especially true for colorectal cancer, a common and possibly largely preventable form of cancer[12].

The effect of dietary inducers may be dependent on genetic makeup. In the coding region of the NQO1 gene, two functional polymorphisms are known, of which one occurs relatively frequently. This C to T sequence variation at cDNA position 609 exists with an allele prevalence of ~20% in Caucasians to ~50% in Asians[13, 14]. It results in lower NQO1 protein stability and enzymatic activity in heterozygotes and its absence in homozygotes for the variant[15-19]. Some studies have reported an increased risk of colorectal tumors associated with the *NQO1* 609T allele[20, 21]. Possibly, *NQO1* 609C>T heterozygotes can compensate for the disadvantage of their dysfunctional allele by consuming NQO1 inducers, and thus enhance overall NQO1 capacity by increasing expression of the functional allele. Polymorphisms that affect NQO1 regulation also may exist; in the regulatory region of the *NQO1* gene itself, or in the gene encoding the transcription factor NFE2L2 (nuclear factor (erythroid-derived 2)-like 2, also known as Nrf2) which acts through the EpRE and is important in the transcriptional activation of the

NQO1 gene[22, 23]. These polymorphisms may act constitutively or their effect may be modulated by consumption of dietary inducers.

In an endoscopy-based population, we investigated the influence of short-term fruit and vegetable consumption and genetic variation in *NQO1* and *NFE2L2* on rectal *NQO1* mRNA expression and NQO1 enzymatic activity. Furthermore, we investigated whether NQO1 activity in white blood cells reflects activity in rectal tissue and could serve as surrogate endpoint.

METHODS

Study population. The study population has been described before[24]. In short, participants were recruited in two outpatient endoscopy clinics in the Netherlands from patients scheduled for a sigmoidoscopy, between January 2003 and June 2004. Eligibility criteria were: age between 18 and 75 years, Caucasian, no chronic inflammatory bowel disease (past or present), no inflammation in the distal colon at the time of endoscopy, no sporadic colorectal cancer (past or present), and no bowel resection. Forty-five percent of invited patients agreed to participate (n=105). Of these, 11 were excluded because inclusion criteria were not met posteriorly. This resulted in a final study population of 94 individuals. The study was approved by the Medical Review Boards of both hospitals. All participants gave their written informed consent.

Medical, dietary and lifestyle information. Medical information was recorded from the endoscopy request form, endoscopy report and, if available, PA report. Participants kept a 3-day dietary record, the third day ending at the time of endoscopy. All records were checked for quality and completeness by the same, trained dietician of the division of Human Nutrition of Wageningen University. Processing into food quantities and coding was done according to the most recent standard manual on food portions and household measures and the Dutch Food Composition Table[25, 26]. Conversion into amounts of nutrients was done using the VBS Food Calculation System[27]. Fruits were subdivided in citrus and non-citrus fruits, vegetables in botanically defined subgroups: Alliaceae (e.g. garlic, leek), Apiaceae (e.g. celery, carrot), Brassicaceae (e.g. cauliflower, broccoli), Compositae (e.g. endive, lettuce), Cucurbitaceae (e.g. zucchini, cucumber), Solanaceae (e.g. bell pepper, tomato; potato not included), and a restgroup.

General lifestyle information was collected through a semi-structured questionnaire containing questions about age, sex, weight, height, smoking habits, medication, disease and family history of cancer.

Specimen collection and preparation. *Biopsies.* Flexible sigmoidoscopy was performed with the patient in left lateral decubitus position. Biopsies

(approximately 20 mg each) were taken from normal rectal mucosa at a distance of 5 to 15 cm from the anal verge. For every subject, two biopsies were collected in RNA stabilization solution (RNA*later*, Qiagen Benelux B.V., Venlo, The Netherlands). All were snap-frozen in liquid nitrogen. *Blood.* Blood (3 x 9 ml) was drawn shortly after endoscopy by venipuncture in Vacuette EDTA K3 Tubes (Greiner Bio-One, Alphen a/d Rijn, the Netherlands). Leukocytes, lymphocytes and buffy coat DNA samples were isolated as described before[24]. *Sample storage.* Rectal biopsies were stored intact at -80°C for 7.5±4.5 months after tissue sampling until homogenation and refreezing; NQO1 enzymatic activity was then measured within 1 month and NQO1 mRNA levels within 2 months. White blood cell pellets were stored intact at -80°C for 6.3±4.5 months after blood sampling until preparation and refreezing. NQO1 activity was then measured within 1 month.

Laboratory assays. *Genotyping*. DNA samples were stored at 4ºC in 8x12 array banks with negative controls. Nomenclature of sequence variation is according to recent consensus[28], but after first mention is made this is abbreviated in the text by omitting 'c.' or 'g.'.

The *NQO1* c.609C>T polymorphism ([16]; position 609 relative to the transcription site of GenBank Accession number J03934) was assessed by PCR-RFLP, with an internal check on digestion (all experimental details available on request). *NQO1* 609C>T genotyping was performed in duplicate and found to be 100% reproducible. It was not in Hardy-Weinberg Equilibrium (HWE): χ^2: 4.26, p-value: 0.039. However, as our genotyping assay has an internal check on complete digestion and was 100% reproducible, we think this statistical deviation from HWE is probably due to a coincident absence of T-homozygotes where three TT-genotypes were expected based on HWE (had there been one TT-genotype, the p-value would have been >0.05).

The *NQO1* g.-718G>A regulatory polymorphism, -718 relative to the transcription initiation site (or -829 relative to the translation start) of GenBank Accession number M81596, genotyped by pyrosequencing the region -725 to -717 of NQO1. We selected this SNP because it was found to have the highest frequency in our sequence survey of ~1100 bp of the NQO1 regulatory region upstream of the transcription initiation site among 96 individuals (unpublished results). The *NQO1* -718G>A genotyping was performed in duplicate and found to be 100% reproducible, and was in HWE (χ^2: 0.32, p-value: 0.57).

The *NFE2L2* (also known as Nrf2) polymorphisms reported by Yamamoto et al.[29], g.-650C>A, g.-684G>A and g.-686A>G relative to the transcription initiation site of GenBank accession number AC079305 (g.-733C>A, g.-767G>A and g.-769A>G, respectively, relative to the translation start of this accession number)

were determined by pyrosequencing. The region -686 to -677 of *NFE2L2* was analyzed in order to genotype the -686A>G and -684G>A polymorphisms, and the region -651 to -646 in order to genotype the -650C>A polymorphism. Genotyping of all three *NFE2L2* polymorphisms was performed in duplicate. One subject could not be genotyped unambiguously for the -686A>G and -684G>A polymorphisms, while another subject could not be genotyped unambiguously for the -650C>A polymorphism. These subjects were excluded from the respective analyses. The other measurements were 100% reproducible. All *NFE2L2* polymorphisms were in HWE (650: χ^2 0.76, p=0.38; 684: χ^2=0.37, p=0.54; 686: χ^2=1.16, p=0.28).

mRNA assays. Rectal biopsies stored in RNA*later* were homogenized on ice using a frozen (-20°C) pestle and subsequently mixed with 1 ml Trizol Reagent (Invitrogen, Breda, The Netherlands). Samples were disintegrated further by passage through one or more syringe needles with decreasing diameter (minimal diameter 0.8 mm). Total RNA was subsequently purified by a standard chloroform-phenol extraction method[30]. RNA concentration was measured spectrophotometrically at 260 nm, and the samples were stored at -80°C until reverse transcription (RT-)PCR using 1-2 μg of total RNA and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) according to the manufacturer's instructions. *NQO1* expression was quantitated by real-time PCR using a SYBR green-based method and using β-actin as a reference. PCR amplification was performed at least in 3-fold. Measurement was not successful for two subjects. *NQO1* mRNA levels were expressed as the *NQO1:β-actin* ratio.

Protein assays. Protein was isolated from rectal biopsies, leukocytes and lymphocytes as described before[24]. Total protein was measured using the Pierce bicinchoninic acid (BCA) protein assay reagent kit (Pierce Rockford, IL, USA) and BSA as a standard, following manufacturer's instructions. To determine NQO1 enzymatic activity, the dicoumarol inhibitable reduction of 2,6-dichlorophenolindophenol (DCPIP, Sigma Aldrich, Zwijndrecht, The Netherlands) was measured spectrophotometrically at 600 nm, as described by Benson et al.[31], with minor modifications[32]. Measurements were performed in duplicate. NQO1 enzymatic activity was normalised to protein content, and expressed as nmol DCPIP reduced/min/mg protein.

Statistical analyses. Excluded from their respective analyses were subjects with: no dietary information (2), no rectal biopsies (2), no mRNA measurement (2), extreme mRNA level (1; NQO1:β-actin ratio of 100.4×10^{-3}), extreme lymphocyte enzyme activity (1; 106 nmol/min/mg protein), and different bowel preparation, which was accompanied by high rectal NQO1 activities (1). Thus, in relation to mRNA level and enzymatic activity, 87 and 90 subjects, respectively, were

available for genotype analyses and 85 and 88, respectively, for fruit/vegetable consumption analyses.

The statistical differences in *NQO1* mRNA level and in NQO1 activity were evaluated between groups based on population characteristics (table 1), consumption of fruits and vegetables (yes/no, tables 2 and 4) and genotype (table 3). Non-parametric testing (Wilcoxon, Kruskal Wallis, savage) was performed, because these methods remain valid for small and skewed sample sizes. A two-sided probability of < 0.05 was considered statistically significant and <0.10 as a signal for possible association. Cells with 1 subject (*NQO1* -718 AA, and *NFE2L2* -650 AA and -684 AA) were not included in the test.

In addition, we explored possibly confounding factors for associations between fruits and vegetables and NQO1 phenotype using linear regression models. Out of the variables age, sex, BMI, mean energy intake, education, time of endoscopy, clinic, sample storage time, smoking, family history of colon cancer, presence of polyps, meat consumption, and coffee consumption, the following univariately showed an association with NQO1 phenotype with a significance of $p=0.10$ or less: endoscopy time and meat consumption for *NQO1* mRNA and smoking and sample storage time for NQO1 activity. Smoking and storage time affected some fruit/vegetable β-estimates by >10%, and were included in the activity models. Endoscopy time and meat consumption did not affect β-estimates and were not included in the mRNA models. Statistical interaction was measured by the addition of a genotype*consumption product term. For power reasons, total fruit and total vegetable consumption groups were based on a median-split of the average intake in g/day here.

Spearman correlation coefficients were calculated to evaluate the association between rectal *NQO1* mRNA level and NQO1 activity, and between rectal and white blood cell NQO1 activities.

Haplotypes were estimated for *NQO1* and *NFE2L2* using the Hplus program version 2.5, available online (Fred Hutchinson Cancer Research Center Hplus. http://qge.fhcrc.org/hplus. The coding and regulatory *NQO1* variants were never found to be linked, and neither were the three *NFE2L2* variants. Variants of both the genes occurred together infrequently. Therefore, all SNPs were analyzed separately.

All statistical analyses were performed using SAS software, version 9.1 (SAS institute, Cary, NC).

RESULTS

Population characteristics. The general and medical characteristics of the population and their relationships with *NQO1* mRNA level and NQO1 enzymatic activity are presented in table 1. Total population mean rectal *NQO1:β-actin* mRNA ratio was $10.2 \times 10^{-3} \pm 5.3 \times 10^{-3}$ (median value 9.4) and mean rectal NQO1 activity was 97 ± 47 nmol DCPIP/min/mg protein (median value 89). *NQO1* mRNA level was lower among those undergoing endoscopy in the morning and NQO1 activity was higher among those with adenomas, both borderline significantly.

Table 1: Rectal NQO phenotype by general study population characteristics

Characteristic			Total	Rectal NQO1:β-actin mRNA ratio median x 10^{3}[a]	Rectal NQO1 activity (nmol DCPIP/ min/mg protein) median
			n=92	n=87	n=90
General characteristics				9.4[a, b]	89[b]
Age	<49.3/>49.3 years[c]		46/46	8.6 / 9.8	88 / 93
Sex	male/female		39/53	8.5 / 9.5	89 / 89
Education	low/high		17/41	7.9 / 9.5	84 / 90
Smoking	never/current/ex		37/18/3	8.2 / 10.5 / 9.7	88 / 94 / 89
BMI	<24.7/>24.7[c]		46/46	9.5 / 8.6	85 / 92
Medical factors[d]					
Family history	cancer	yes/no	48/43	8.5 / 9.8	90 / 87
	colorectal cancer	yes/no	7/85	7.9 / 9.5	85 / 89
Indication for endoscopy[e]	blood loss per anum	yes/no	44/48	8.5 / 9.8	84 / 93
	abdominal pain	yes/no	30/62	8.8 / 9.5	89 / 88
	different defecation pattern	yes/no	30/62	9.8 / 9.1	91 / 89
Endoscopy characteristics[f]					
Outpatient clinic	A/B		79/13	9.4 / 8.1	88 / 94
Endoscopy time	morning/afternoon		48/44	8.4 / 10.6*	85 / 89
Macroscopic view	hemorrhoids	yes/no	17/74	9.7 / 9.4	90 / 89
	diverticula	yes/no	13/78	9.7 / 9.4	84 / 89
	adenoma(s)	yes/no	10/81	10.3 / 9.4	102 / 88*

[a]e.g., 9.4 means a NQO1:β-actin mRNA ratio of 0.0094
[b]mean ± sd: 10.2 ± 5.3 and 97 ± 47 for mRNA and activity, respectively
[c]median split; age: mean±sd = 46.5±13.8; BMI: mean±sd = 25.5±4.6
[d]source: medical record and self-reported
[e]the three most common indications are shown; more than one possibility per individual
[f]bowel preparation, day before: 17:00 hrs: 2 laxatives (10 mg), 20:00 hrs microlax enema (5 ml)
*p<0.10 (the difference between the characteric-groups was tested by Wilcoxon test)

First, the influence of recent fruit and vegetable consumption on *NQO1* mRNA level and NQO1 activity (not considering genotype) is presented, then the influence of genetic polymorphisms in *NQO1* and *NFE2L2* on these parameters are described, followed by their possible interaction in determining NQO1 phenotype.

Rectal NQO1 phenotype by fruit and vegetable consumption. Mean total fruit and mean total vegetable consumption among consumers on the 2 days preceding endoscopy was 144 ± 116 and 128 ± 89 g/day, respectively (table 2). Total consumption of fruits (yes/no) was not significantly associated with rectal *NQO1* mRNA or NQO1 activity. Vegetable consumers did appear to have lower *NQO1* mRNA levels. Consumption of Compositae (yes/no) was inversely associated with *NQO1* mRNA level (table 2); mean *NQO1:β-actin* mRNA ratio was 2.7 x 10^{-3} (95% confidence interval (CI): 0.45 x 10^{-3} , 5.0 x 10^{-3}) lower among consumers as compared to non-consumers. Consumption of Apiaceae (yes/no) was inversely associated with rectal NQO1 activity. Adjusted for smoking and sample storage time, it was 23 nmol DCPIP/min/mg protein (95% CI: 1.4, 45) lower among consumers as compared to non-consumers (data not in table).

Table 2: Rectal NQO1 phenotype by fruit and vegetable consumption (yes/no)

Consumption		Intake, g/day[a]		Rectal NQO1:β-actin mRNA ratio		Rectal NQO1 activity (nmol DCPIP/ min/mg protein)	
		n	mean ± sd (median)	n	mean ± sd (median) x10^{3}[b]	n	mean ± sd (median)
Total population		88	225 ± 159 (200)	85	10.2 ± 5.3 (9.4)[b]	88	97 ± 47 (89)
Total fruit	No	20	-	20	10.8 ± 4.3 (10.2)	20	105 ± 59 (93)
	Yes	68	144 ± 116 (120)	65	10.1 ± 5.6 (9.4)	68	94 ± 43 (88)
Citrus fruit[c]	No	62	-	61	10.1 ± 5.4 (8.7)	62	100 ± 52 (90)
	Yes	26	84 ± 59 (60)	24	10.8 ± 5.3 (10.1)	26	89 ± 33 (83)
Total vegetables[d]	No	10	-	10	13.3 ± 8.8 (11.8)	10	94 ± 42 (85)
	Yes	78	128 ± 89 (113)	75	9.9 ± 4.6 (9.4)**	78	97 ± 48 (89)
Alliaceae	No	52	-	51	10.3 ± 6.0 (9.4)	52	93 ± 46 (86)
	Yes	36	28 ± 28 (21)	34	10.3 ± 4.4 (9.1)	36	103 ± 50 (90)
Apiaceae	No	63	-	61	10.5 ± 5.5 (9.4)	63	103 ± 53 (89)
	Yes	25	50 ± 43 (44)	24	9.8 ± 5.1 (9.1)	25	81 ± 26 (86)*
Brassicaceae	No	60	-	58	9.9 ± 5.6 (8.6)	60	96 ± 51 (88)
	Yes	28	53 ± 38 (40)	27	11.2 ± 4.7 (10.0)	28	98 ± 38 (91)
Compositae	No	51	-	50	11.4 ± 5.7 (10.3)	51	98 ± 44 (88)
	Yes	37	50 ± 37 (38)	35	8.7 ± 4.4 (8.2)**	37	96 ± 52 (89)
Cucurbitaceae	No	59	-	58	10.7 ± 5.8 (9.6)	59	94 ± 47 (88)
	Yes	29	44 ± 30 (40)	27	9.4 ± 4.1 (8.5)	29	102 ± 48 (93)
Solanaceae	No	58	-	57	10.7 ± 5.7 (8.7)	58	101 ± 49 (89)
	Yes	30	53 ± 45 (34)	28	9.5 ± 4.5 (9.5)	30	88 ± 44 (81)

[a]average intake on the 2 days preceding endoscopy, 2 food records missing

[b]e.g., 10.3 means a NQO1:β-actin mRNA ratio of 0.0103

[c]results are similar for combined group of citrus fruit and juice; juice consumption among 21 consumers: 176 ± 117 g/d

[d]does not include potato

**p<0.05, *p<0.10 (the difference between the yes vs no consumption groups was tested by Wilcoxon and Savage test)

Rectal NQO1 phenotype by *NQO1* and *NFE2L2* genotype. The *NQO1* 609CT genotypes showed a higher *NQO1* mRNA level than the 609CC genotypes (figure 1a, table 3); the mean difference in *NQO1:β-actin* mRNA ratio amounted to 3.9 x 10^{-3} (95% CI: 1.7 x 10^{-3}, 6.1 x 10^{-3}). NQO1 activity was lower among *NQO1* 609CT genotypes as compared to 609CC genotypes (figure 1b, table 3); the mean difference amounted to 39 nmol DCPIP/min/mg protein (95% CI: 21, 58). The *NQO1* mRNA level, but not NQO1 activity, was higher among the *NFE2L2* -684GA genotypes than among the -684GG genotypes (table 3), the two genotypes showing a difference of 2.6 x 10^{-3} (95% CI: 0.034 x 10^{-3}, 5.1 x 10^{-3}). The *NQO1* -718 G>A regulatory polymorphism and other *NFE2L2* polymorphisms did not affect rectal *NQO1* mRNA level or NQO1 activity significantly (table 3).

Table 3: Rectal NQO1 phenotype by *NQO1* and *NFE2L2* genotype

Genotype		Total	Rectal NQO1:β-actin mRNA ratio			Rectal NQO1 activity (nmol DCPIP/ min/mg protein)
		n	n	mean±sd (median) x10^{3a}	n	mean±sd (median)
All genotypes		92	87	10.2 ±5.3 (9.4) [a]	90	97±47 (89)
NQO1	CC	59	55	8.8 ± 4.3 (7.9)	58	110 ± 51 (96)
c.609C>T	CT	33	32	12.7 ± 6.0 (11.2)**	32	72 ± 23 (73)***
	TT	0	0	-	0	-
NQO1	GG	78	74	10.6 ± 5.6 (9.5)	77	93 ± 42 (88)
g.-718G>A	GA	13	12	8.1 ± 2.8 (8.0)	12	113 ± 73 (92)
	AA	1	1	6.2	1	144
NFE2L2	CC	79	76	10.2 ± 5.3 (9.1)	77	98 ± 46 (89)
g.-650C>A	CA	11	9	10.0 ± 5.7 (9.5)	11	78 ± 25 (84)
	AA	1	1	11.7	1	198
NFE2L2	GG	67	63	9.5 ± 5.5 (8.4)	66	97 ± 48 (89)
g.-684G>A	GA	23	22	12.1 ± 4.2 (13.1)*	22	94 ± 48 (86)
	AA	1	1	15.2	1	93
NFE2L2	AA	35	31	9.8 ± 4.6 (9.5)	33	85 ± 37 (84)
g.-686A>G	AG	47	46	10.4 ± 5.8 (8.6)	47	103 ± 46 (93)
	GG	9	9	11.1 ± 5.5 (9.7)	9	107 ± 79 (80)

[a]e.g., 10.2 means a NQO1:β-actin mRNA ratio of 0.0102

*p<0.05, **p<0.001, ***p<0.0001 (the difference between genotypes was tested by Wilcoxon test for two groups and Kruskal Wallis test for three groups; cells with 1 observation were not included in the test)

Figure 1: Rectal NQO1 phenotype by *NQO1* c.609C>T genotype

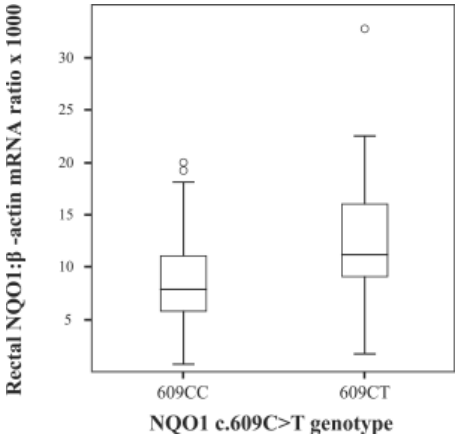

Figure 1a: Rectal *NQO1* mRNA level by *NQO1* c.609C>T genotype

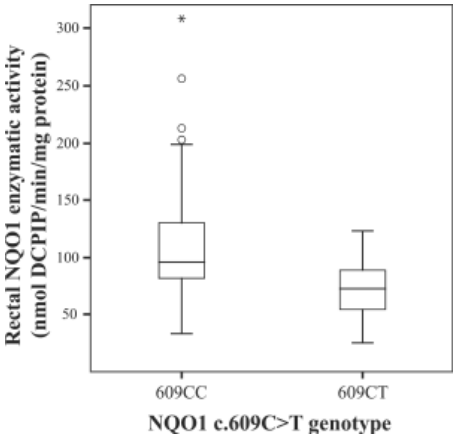

Figure 1b: Rectal NQO1 enzymatic activity by *NQO1* c.609C>T genotype

Footnote
Boxes and bars represent the interquartile range and the median value, respectively; Whiskers represent distance to The smallest and largest sample value, excluding outliers; ○ and * represent values of 1.5-3 and > box lengths from the box, resp.

When stratified by the *NQO1* 609C>T polymorphism (table 4), the difference in *NQO1:β-actin* mRNA ratio between the *NFE2L2* -684GA and GG genotypes was visible among the *NQO1* 609CC genotypes (2.9×10^{-3}; 95% CI: 0.1×10^{-3}, 5.6×10^{-3}; non-parametric test p=0.059) and not among the 609CT genotypes (0.85×10^{-3}; 95% CI: -4.1×10^{-3}, 5.8×10^{-3}), but this was not a significant interaction (p for interaction = 0.42). The lack of association between the *NFE2L2* -684G>A polymorphism and NQO1 enzymatic activity remained after stratification by the *NQO1* 609C>T polymorphism. There also were no significant differences with regard to NQO1 phenotype between the *NQO1* -718 G>A genotypes or the other *NFE2L2* genotypes after stratifying for the *NQO1* 609C>T genotype.

Overall, the Spearman correlation coefficient between rectal *NQO1* mRNA level and NQO1 activity was 0.14 (p=0.20). The strength of this correlation differed by *NQO1* 609C>T genotype. Among *NQO1* 609CC genotypes, the Spearman correlation coefficient between *NQO1* mRNA level and NQO1 activity was 0.50 (p=0.0001) and among 609CT genotypes it was 0.14 (p=0.45).

Table 4: Rectal NQO1 phenotype by the combined *NQO1* 609C>T and *NFE2L2* genotypes

		Rectal NQO1:β-actin mRNA ratio				Rectal NQO1 activity (nmol DCPIP/min/mg protein)			
		NQO1 c.609 CC mean±sd		*NQO1* c.609 CT mean±sd		*NQO1* c.609 CC mean±sd		*NQO1* c.609 CT mean±sd	
		n	(median) x10[3a]	n	(median) x10[3a]	n	(median)	n	(median)
NFE2L2	CC	52	8.8 ± 4.4 (7.9)	24	13.0 ± 6.1 (12.2)	53	110 ± 51 (95)	24	71 ± 25 (67)
g.-650C>A	CA	2	6.1 ± 1.6 (6.1)	7	11.1 ± 6.0 (10.0)	4	88 ± 35 (80)	7	73 ± 19 (84)
	AA	1	11.7	0	-	1	198	0	-
NFE2L2	GG	43	8.2 ± 4.1 (7.6)	20	12.5 ± 7.1 (10.9)	46	109 ± 51 (95)	20	71 ± 25 (76)
g.-684G>A	GA	12	11.0 ± 4.6 (11.2)*	10	13.3 ± 3.5 (13.9)	12	116 ± 54 (105)	10	67 ± 18 (63)
	AA	0	-	1	15.2	0	-	1	93
NFE2L2	AA	16	8.7 ± 4.0 (8.3)	15	10.9 ± 5.0 (9.9)	18	98 ± 42 (89)	15	70 ± 23 (78)
g.-686A>G	AG	31	8.4 ± 4.3 (7.5)	15	14.5 ± 6.6 (13.6)	32	117 ± 47 (103)	15	72 ± 23 (68)
	GG	8	10.2 ± 5.1 (8.7)	1	18.0	8	112 ± 83 (80)	1	66

*p<0.10, (the difference between *NFE2L2* genotypes was tested by Wilcoxon test for two groups and Kruskal Wallis test for three groups; cells with 1 observation were not included in the test)

Rectal NQO1 phenotype by total fruit and vegetable consumption and by *NQO1* and *NFE2L2* genotype. Total fruit and vegetable consumption as divided in median groups was not statistically significantly associated with *NQO1* mRNA level or NQO1 activity (table 5). No statistically significant differences were observed when taking genotype into account, with the exception of the *NFE2L2* -686A>G genotype in relation to NQO1 activity; High consumption of fruits and vegetables was associated with a lower NQO1 activity among the *NFE2L2* -686AA and -686GG genotypes, but with a higher activity among the -686AG genotypes (table 5).

When considering total fruit consumption only (data not in table), there was a significant statistical interaction between the *NQO1* 609C>T polymorphism and *NQO1* mRNA level (p for interaction = 0.040); Among 609CC-genotypes, high consumption of fruit was associated with a 2.9×10^{-3} (95% CI 0.61×10^{-3}, 5.2×10^{-3}) lower *NQO1:β-actin* mRNA ratio as compared to low consumption. Among CT-genotypes, the ratio was higher with high fruit consumption, but not significantly: 1.7×10^{-3} (95% CI -2.7×10^{-3}, 6.1×10^{-3}).

Total vegetable consumption only, as divided in median groups, was not associated with *NQO1* mRNA level or NQO1 activity (data not in table), but there was statistical interaction between vegetable consumption and the *NQO1* -718G>A polymorphism in NQO1 activity, showing similar data as for the total fruits and vegetable groups in table 5. Also, there was a borderline significant interaction between vegetable consumption and the *NFE2L2* -650C>A polymorphism. Looking further into vegetable subtypes, consumption of Compositae (yes/no) was significantly inversely associated with rectal *NQO1:β-actin* mRNA ratio among *NFE2L2* -650CC genotypes: -3.2×10^{-3} (95% CI -5.6×10^{-3}, -0.78×10^{-3}) as compared to non-consumption, but not among -650CA genotypes (2.0×10^{-3}; -7.5×10^{-3}, 11.5×10^{-3}; data not shown in table). Consumption of Compositae (yes/no) was significantly inversely associated with rectal NQO1 activity among *NFE2L2* -686AA genotypes: -31 nmol DCPIP/min/mg protein (95% CI -58, -5.0) as compared to non-consumption, but not among –686AG and GG genotypes: 5.4 (-24, 35) and 8.0 (-19,35) nmol DCPIP/min/mg protein, respectively; data not shown in table). Results of interactions between genotypes and consumption of fruits and/or vegetables need to be considered with caution, as numbers are small and the influence of single observations can be quite high.

Table 5: Rectal NQO phenotype by fruit and vegetable consumption (high/low) and by *NQO1* and *NFE2L2* genotype

		Rectal NQO1:β-actin mRNA ratio				Rectal NQO1 activity (nmol DCPIP/min/mg protein)			
Fruit & vegetable consumption		low consumption[a] (98±62 g/d, 101 g/d) mean±sd		high consumption[a] (358±137 g/d, 303 g/d) mean±sd		low consumption[a] (98±62 g/d, 101 g/d) mean±sd		high consumption[a] (358±137 g/d, 303 g/d) mean±sd	
Genotype		n	(median)*10^{-3}[b]	n	(median)*10^{-3}	n	(median)	n	(median)
All genotypes		43	11.0 ± 5.8 (10.4)[b]	42	9.5 ± 4.8 (9.0)	44	104 ± 55 (91)	44	89 ± 37 (85)
NQO1	CC	26	9.5 ± 4.8 (7.8)	27	8.1 ± 3.8 (7.9)	27	125 ± 60 (103)	29	99 ± 39 (89)
c.609C>T	CT	17	13.3 ± 6.5 (13.6)	15	12.0 ± 5.5 (9.9)	17	72 ± 22 (68)	15	71 ± 24 (81)
	TT	0	-	0	-	0	-	0	-
NQO1	GG	38	11.2 ± 6.1 (10.2)	34	10.2 ± 5.0 (9.5)	39	99 ± 45 (91)	36	88 ± 38 (84)
g.-718G>A	GA	5	9.8 ± 1.9 (10.8)	7	6.9 ± 2.9 (7.0)	5	148 ± 105 (96)	7	88 ± 28 (89)
	AA	1	6.2	0	-	1	144	0	-
NFE2L2	CC	36	11.2 ± 5.9 (10.6)	38	9.3 ± 4.7 (8.6)	36	106 ± 57 (93)	39	92 ± 38 (85)
g.-650C>A	CA	5	8.9 ± 5.7 (7.7)	4	11.3 ± 6.2 (10.2)	6	83 ± 32 (85)	5	73 ± 16 (62)
	AA	1	11.7	0	-	1	198	0	-
NFE2L2	GG	31	10.4 ± 6.2 (8.6)	32	8.7 ± 4.8 (8.3)	32	103 ± 55 (90)	34	92 ± 40 (85)
g.-684G>A	GA	11	12.4 ± 4.4 (14.0)	9	12.7 ± 3.9 (12.9)	11	109 ± 61 (93)	9	78 ± 22 (84)
	AA	1	15.2	0	-	1	93	0	-
NFE2L2	AA	14	9.9 ± 4.8 (10.5)	16	9.7 ± 4.7 (9.4)	14	105 ± 43 (92)	18	68 ± 23 (69)*
g.-686A>G	AG	27	11.4 ± 6.2 (10.4)	18	9.2 ± 5.2 (8.2)	28	97 ± 49 (89)	18	114 ± 38 (106)
	GG	2	13.8 ± 8.6 (13.8)	7	10.3 ± 4.9 (9.7)	2	205 ± 145 (205)	7	79 ± 25 (78)^

[a]median value on which low and high fruit and vegetable consumption groups are based: 199.5 g; does not include juice

[b]e.g., 11.0 means a NQO1:β-actin mRNA ratio of 0.011

*p<0.05 (the difference between the high vs low consumption groups was tested by Wilcoxon test)

^p for interaction <0.01 (calculated as departure from multiplicativity using linear regression; enzymatic activity was adjusted for smoking and sample storage time)

NQO1 activity in white blood cells. For comparison with rectal NQO1 activity, NQO1 enzymatic activity was also measured in total leukocytes (n=73) and in lymphocytes (n=91). It amounted to a mean of 19.9 ± 5.5 and 23.9 ± 5.8 nmol DCPIP/min/mg protein, respectively. There was no correlation between NQO1 activity in rectal biopsies and NQO1 activity in total leukocytes (r=0.10, p=0.40), or lymphocytes (r=-0.02, p=0.88). This absence of correlation did not change when stratifying for the *NQO1* 609C>T polymorphism. There also was no correlation between total leukocyte and lymphocyte NQO1 activity (r=0.003, p=0.98).

White blood cell NQO1 activity did not appear to be subject to the same genetic and/or exogenous influences as rectal NQO1 activity. There was no influence from recent fruit and vegetable consumption on lymphocyte activity and only consumption of Alliaceae was positively associated with leukocyte activity (non-parametric p=0.0425; adjusted β-estimate 2.15 nmol DCPIP/min/mg protein (95% CI -0.18, 4.47). The *NQO1* 609CT genotypes had somewhat lower NQO1 activity in leukocytes and lymphocytes than the CC genotypes, but the difference was not significant: -1.8 (95% CI -4.1, 0.52) and -1.03 (-3.49, 1.45) DCPIP nmol/min/mg protein, respectively.

DISCUSSION

In this endoscopy-based population genetic variation, in particular the *NQO1* 609C>T polymorphism, appeared more relevant for rectal *NQO1* mRNA level and NQO1 enzymatic activity than fruit and vegetable consumption. White blood cell NQO1 activity did not reflect rectal activity.

Consumption of fruits and vegetables did not show the expected positive association with NQO1 phenotype, but rather there appeared to be an inverse association with *NQO1* mRNA and NQO1 activity. With regard to specific subtypes, consumption of Compositae (yes vs no) was associated with lower rectal *NQO1* mRNA level and consumption of Apiaceae (yes vs no) was associated with lower rectal NQO1 activity. One factor that may play a role here is the relatively high content of pro-vitamin A carotenoids of Apiaceae and Compositae[33]. These can be metabolised to retinoic acid (RA), which was found to have a repressive effect on GSTP1 expression, mediated by an AP-1-binding site (TGACTCA) in the GSTP1 regulatory region[34]. An AP-1 sequence is also embedded in the NQO1 EpRE[22] and this AP-1 sequence was shown to be functional, since it was found to bind transcription factor AP-1[35] leading to repression of NQO1 transcription[36]. Moreover, the expression of GSTA and GSTM2 protein, which both have an AP-1 binding site in their regulatory region[37] was also found to be diminished upon Apiaceae consumption[24, 38]. Therefore, AP-1 binding site-mediated repression of

NQO1 transcription by RA may play a role in the observed inhibitory effect of Apiaceae and Compositae on *NQO1* mRNA transcription and NQO1 enzyme activity.

Consumption of known inducers of human *NQO1* mRNA or NQO1 protein, such as Allium or Brassica vegetables[6, 39, 40], was not significantly associated with higher *NQO1* mRNA expression and/or NQO1 activity, although individuals who consumed Brassica vegetables did show higher *NQO1* mRNA levels. The purpose of our food record was to estimate actual consumption on the two days prior to endoscopy (and not habitual consumption), because the transcriptional process occurs rapidly, in a matter of hours to days after consumption of the inducer[6, 41] and therefore the time window should have been sufficient to show any possible effects of the measured fruit and vegetable consumption. Perhaps, their inducing effects are overruled by other components of the total diet. Because of our observational design, most subjects were exposed to more than one type of fruit or vegetables. However, as we eat total diets and not components, this does reflect relevant exposure. Fruit intake was similar to and vegetable intake and percentage of vegetable consumers somewhat lower than that reported in the 1998 Dutch National Food Consumption Survey, a 2-day dietary record among representatives of the Dutch population[42]. Possibly, the exposure to some fruit and vegetable components was too low to show significant differences in NQO1 phenotype. E.g., most intervention studies that show inducing effects of Brassica vegetables on NQO1 or GST (to which a similar mechanism applies) phenotype use doses of ~300 grams[6, 38, 43, 44]. Altogether, our study results indicate a limited role for recent regular fruit and vegetable consumption in human NQO1 phenotype.

A role for fruits and vegetables may also be overruled by the *NQO1* 609C>T polymorphism, which was the most significant determinant of both *NQO1* mRNA expression and NQO1 activity. In this study, the genotype distribution of the *NQO1* 609C>T polymorphism was comparable to frequencies found in other Northern European studies[45, 46]. The distribution of the *NQO1* -718G>A polymorphism was comparable to that reported on the NCBI-website under rs689457 for a population with European ancestry[47]. As far as we know, the distributions of the three *NFE2L2* polymorphisms have not been reported before for a (Northern) European population.

We confirmed, in rectal biopsies, that NQO1 activity is lower among *NQO1* 609 heterozygotes. This is due to the C to T change leading to a proline to serine amino acid substitution, which results in rapid degradation of the variant protein and thus rapid loss of activity[48]. The correlation coefficient between *NQO1* mRNA level and NQO1 activity was low. The lower enzymatic activity not preceded by

lower mRNA level among those with the *NQO1* 609T-allele partly explains this low correlation coefficient.

Interestingly, while rectal NQO1 activity was lower, rectal *NQO1* mRNA expression was higher among individuals with the NQO1 609CT-genotype as compared to the 609CC-genotype. A similar observation has been reported for lung tissue[49]. A kind of feed-back control of the possible physiological consequences of the non-functional *NQO1* 609T-allele, i.e. higher levels of oxidative stress, may play a role in this. This could result in increased activation of transcription factor NFE2L2, which is considered to be responsive to intracellular oxidative stress[23] and the major transcription factor involved in EpRE-mediated NQO1 expression control.

Another explanation for the higher mRNA expression with the *NQO1* CT-genotype could be the existence of a uninducible C-allele, as proposed by Begleiter et al. based on studies of human tumor cell lines using 1,2-dithiol-3-thione (D3T), a known synthetic NQO1 inducer naturally occurring in e.g. cruciferous vegetables. Some *NQO1* 609CC cell lines in their studies were not inducible by D3T and in some *NQO1* 609CT cell lines there was induction of variant protein (i.e. encoded by the T-allele), only[50]. This phenomenon could also obscure an association between plant food inducers and activity. However, so far, a common genetic difference in the *NQO1* promoter causing a clear difference in NQO1 regulation has not been identified, neither in a subsequent study by Begleiter et al.[51] nor by our work.

The correlation between *NQO1* mRNA and NQO1 activity was low, also among the *NQO1* 609CC genotypes (r=0.50). It is important to consider both mRNA and activity, and their determinants, because mRNA is the first measure of induction, but activity is ultimately the more functional parameter for health. For practical reasons, biopsies for the mRNA and protein measurements were sampled and processed separately. The possible differences in sampling may have contributed to the low mRNA – activity correlation[3]. A regulatory mechanism that could further explain the low correlation between *NQO1* mRNA and NQO1 activity is alternative splicing. This is a common process in higher eukaryotes and may serve as a control mechanism for *NQO1* expression[52]. One specific alternatively spliced transcript lacks exon 4, which contains the quinone binding site, and therefore lacks activity[53, 54]. It has been associated with the low-frequency *NQO1* 465C>T polymorphism (~5%) at position -3 before the 5′-splice site of intron-4[14, 52]. We did not discriminate between variant and full-length transcripts in our mRNA assay, and thus cannot quantitate the effect of its presence in our population. In the study by Szarka et al., its contribution differed between individuals, but in general was not high in colon mucosa[55].

There may be a functional role for genetic variation in transcription factor NFE2L2. The *NFE2L2* -684G>A polymorphism was positively associated with rectal *NQO1* mRNA expression (though this was not translated into a difference in activity) and warrants further investigation. Due to the relatively strong influence of the *NQO1* 609C>T polymorphism and lower power when stratifying for this polymorphism, a role for *NFE2L2* polymorphisms in NQO1 phenotype is difficult to evaluate. Another complicating matter is that the functioning of the protein that regulates NFE2L2 activity (Keap1) could also be subject to genetic variation[56]. Altogether, this multitude of factors illustrates the complexity and diversity of cancer-biochemopreventive pathways.

Although sample size was small for detecting more complex interactions, our study's power was on average (with group sizes varying) >85 % to detect a 50% difference in NQO1 phenotype. As no experimental data are available concerning the functionality of the *NQO1* regulatory region and the *NFE2L2* polymorphisms in rectal enzyme phenotype, their reported interactions need to be regarded with caution. Of course, with multiple comparisons there is also a risk of chance findings. To elucidate the determinants of rectal NQO1 activity, larger studies, at least stratified for the *NQO1* 609C>T polymorphism and discriminating between differences in exposure to inducers and repressors, are needed.

Because rectal sampling is invasive and impractical, and because activity (and not mRNA level) eventually is the most relevant endpoint when considering protection against cancer, we examined if NQO1 activity in white blood cells may be a good surrogate for rectal activity. This does not appear to be the case. O'Dwyer et al. observed a close correlation for *NQO1* mRNA expression between peripheral mononuclear cells and sigmoid samples, and conclude that colonic *NQO1* gene expression can be monitored by using peripheral mononuclear cells[8]. However, *NQO1* mRNA level does not necessarily predict NQO1 protein level or activity. The lack of correlation between white blood cell and rectal NQO1 activity in our study may reflect the complexity of influences (both genetic and exogenous) on the resulting NQO1 capacity in different parts of the body.

Overall, the results of the present study demonstrate that both rectal *NQO1* mRNA level and NQO1 enzymatic activity are influenced most by the *NQO1* 609C>T polymorphism. The *NFE2L2* -684G>A polymorphism possibly affects *NQO1* mRNA expression. Recent fruit and vegetable consumption may repress rather than induce rectal NQO1 phenotype. White blood cell NQO1 activity is not a good surrogate parameter for rectal NQO1 activity. Thus, to better understand the effects of fruits and vegetables in the large intestine, genetic variation needs to be taken into consideration in larger, tissue-specific studies.

ACKNOWLEDGEMENTS

The authors wish to thank Petra Vissink and Els Siebelink for the dietary assessment; and Elgin Lichtenauer for development of the NQO1 609 C>T genotyping assay; Lucy Okma, Annie van Schaik and René te Morsche for lab-related support; the endoscopy staff of the Radboud University Nijmegen Medical Center and the Canisius-Wilhelmina Hospital Nijmegen for their support in recruitment; and all study subjects for their kind participation.

This work was supported by the Netherlands Organisation for Health Research and Development (ZonMW), grant number: 21000054, and the Dutch Digestive Diseases Foundation (MLDS), grant number WS 00-31.

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Abstract

Both environment and genetics contribute to the pathogenesis and prevention of colorectal neoplasia. NAD(P)H:quinone oxidoreductase (NQO1) is a detoxification enzyme that is polymorphic and inducible. We investigated interactions between lifestyle factors and polymorphisms in NQO1 and its key regulatory transcription factor NFE2L2 in colorectal adenoma risk. The *NQO1* c.609C>T and g.-718A>G and *NFE2L2* g.-650C>A, g.-684G>A and g.-686A>G polymorphisms were determined among 740 Dutch adenoma cases and 698 endoscopy-based controls. Dietary intake was assessed by FFQ, other lifestyle information by questionnaire. The *NQO1* 609CT genotype was associated with a higher adenoma risk (OR 1.27, 95% CI 1.00-1.62) compared to the 609CC genotype, whereas the 609TT genotype was not (OR 1.03, 95% CI 0.56-1.88). The higher risk with the *NQO1* 609CT-genotype was seen among smokers (OR 1.96, 95% CI 1.40-2.76), but not among non-smokers (OR 0.91, 95% CI 0.62-1.35; interaction p=0.030). Fruit and vegetable consumption did not protect smokers from adenomas and did not interact with the *NQO1* 609C>T polymorphism or the *NFE2L2* polymorphisms. A higher adenoma risk seen with high fruit and vegetable consumption among *NQO1* -718GG genotypes was absent among -718GA genotypes (interaction p=0.071). Gene-gene interactions were observed between the *NQO1* 609C>T and *NFE2L2* -686A>G polymorphisms (interaction p=0.056) and between the *NQO1* -718 G>A and *NFE2L2* -650C>A polymorphisms (interaction p=0.013). In conclusion: the *NQO1* 609CT genotype is associated with increased adenoma risk among smokers, which is not diminished by high fruit and vegetable consumption. The observed gene-gene interactions may point to a role for *NFE2L2* polymorphisms in NQO1-related adenoma formation.

Chapter 5

NQO1 and *NFE2L2* polymorphisms, fruit and vegetable intake and smoking, and the risk of colorectal adenomas in an endoscopy-based population

Mariken J. Tijhuis
Marleen H.P.W. Visker
Jac M.M.J.G. Aarts
Wijnand Laan
Sybrand Y de Boer
Frans J. Kok
Ellen Kampman

International Journal of Cancer, in press

INTRODUCTION

Both environmental and genetic factors contribute to the pathogenesis as well as prevention of human colorectal neoplasia. The colorectal area is exposed to a wide variety of carcinogens and protective compounds. An important biotransformation enzyme, that may play a role in the detoxification of dietary and other carcinogens, is NAD(P)H:quinone oxidoreductase (NQO1, EC 1.6.5.2)[1].

NQO1 is subject to genetic variation[2]. Two functional single nucleotide polymorphisms (SNPs) have been reported: the 609 C to T substitution with a prevalence ranging from ~20% in Caucasians to ~50% in Asians and the 465 C to T substitution with low prevalence[2]. The 609C>T SNP translates into an amino acid change and results in loss of enzyme activity[3-5]. Its consequences for colorectal cancer risk are not clear. A meta-analysis of 6 studies (5 in Caucasians and 1 in Asians) concluded that the heterozygous genotype (609CT) was associated with a modestly higher colorectal cancer risk (OR 1.15)[6]. The homozygous variant genotype (609TT), however, was not significantly associated with cancer risk and even appeared to be protective in Asians[6]. With respect to colorectal adenomas, intermediates in colorectal carcinogenesis, an increased risk has been reported with the 609CT genotype only in one study[7] and with the 609TT genotype only in another study[8].

Environmental exposure to anticarcinogens and procarcinogens is relevant for the risk assessment of the *NQO1* 609C>T SNP. NQO1 may play a role in the colorectal biotransformation of cigarette smoke[7-10], alcohol[7], meat[11] and may be inducible by coffee, polycyclic aromatic hydrocarbons (PAHs) and various other environmental compounds[12-14]. Also, NQO1 is thought to be inducible by various fruit and vegetable components[12-16]. High exposure to inducers such as fruits and vegetables could be beneficial for individuals with the *NQO1* 609CT genotype as it may upregulate their one functioning (C) allele, without concomitant (pro)carcinogen exposure. The relation between the *NQO1* 609C>T SNP and high fruit or high vegetable consumption in colorectal cancer risk has rarely been investigated, however, and a risk difference between *NQO1* 609 coding region variants has not been observed[11]. In this respect, the regulatory region of the *NQO1* gene is also relevant, as it contains sequences such as the electrophile responsive element (EpRE) through which inducers act to promote transcription. So far no genetic variation has been described in the EpRE of the *NQO1* gene, but polymorphisms may exist in other *NQO1* regulatory region nucleotide sequences, which may also turn out to be functional. EpRE-controlled transcription involves binding of transcription factor nuclear factor (erythroid-derived 2)-like 2 (NFE2L2, also known as Nrf2). For the *NFE2L2* gene, SNPs have also been reported[17], but

little is known about their functionality in human populations. These SNPs may influence the regulation of NQO1 and could thus affect NQO1-related differences in adenoma risk.

The aim of this study was to explore the associations between genetic variation in *NQO1* and *NFE2L2*, exposure to environmental factors (i.e. cigarette smoking, meat consumption and alcohol consumption) and the possible beneficial role of fruit and vegetable consumption, in colorectal adenoma risk.

METHODS

Population. Study design and population have been described before[18, 19]. Cases and controls were recruited among those undergoing endoscopy in 10 endoscopy outpatient clinics in the Netherlands between June 1997 and October 2002. Eligibility criteria were: Caucasian, Dutch speaking, age 18-75 years at time of endoscopy, no hereditary colorectal cancer syndromes, no chronic inflammatory bowel disease, no history of colorectal cancer and no (partial) bowel resection. Most common endoscopy indications were bowel discomfort, change in defecation pattern, anal bleeding or, for cases, follow-up after earlier adenoma. Cases had at least one histologically confirmed colorectal adenoma ever in their lifetime. Controls had no medical history of any type of polyp, confirmed by full colonoscopy (75%) or sigmoidoscopy combined with X-ray (10%). Fifteen percent of subjects did not have full visualisation of the colon, i.e. they had a sigmoidoscopy without X-ray or colonoscopy where the caecum was not reached.

The study was approved by the Medical Review Boards of all participating hospitals and of Wageningen University. The overall response rate was estimated to be 55%, varying from 35% to 90% between outpatient clinics. The total study population consisted of 1477 participants (768 cases and 709 controls). All gave their written informed consent.

Diet and other lifestyle factors. Dietary intake was assessed by a self-administered food frequency questionnaire (FFQ) developed for the Dutch European Prospective Investigation Into Cancer cohort and processed using the Dutch food composition table[20]. Frequency of consumption could be indicated per day, week, month or year. Portion sizes were estimated from color photographs. Frequencies and portion sizes were multiplied to obtain grams for each food item. Total vegetable consumption was calculated as the sum of raw and cooked individual vegetables and total fruit consumption as the sum of individual fruits and corrected proportionally to the frequency reported on total vegetables and on total fruits[20]. Total vegetables and total fruits were summed into one variable to reflect the composite exposure to its many phytochemicals. Total meat

consumption was calculated according to Linseisen et al.[21] as the sum of fresh meat (among which fresh red meat) and processed meat; Fresh red meat was defined as beef, pork and other; Processed meat was defined as cold/sandwich meats, sausage and meat snacks. Alcoholic beverages were divided in beer, white wine, red wine, ports and liquors, and ethanol consumption was measured as the number of glasses multiplied by its ethanol content (~ 10 grams). Coffee consumption was measured as the number of cups consumed. The FFQ referred to habitual intake in the year preceding endoscopy or bowel problems. General lifestyle factors, such as smoking behaviour, were assessed by a structured self-administered questionnaire. Both FFQ and general questionnaire were handed at the time of endoscopy or sent within three months after endoscopy. Endoscopy-related and other medical information was abstracted from the patient's medical record.

Genotyping. EDTA treated whole blood was used for DNA extraction (QIAamp 96 DNA blood kit, Qiagen Inc.). DNA was stored at 4ºC in 8*12 array banks with negative controls. Laboratory staff was blinded for case-control status. Nomenclature of sequence variation is according to recent consensus[22], but after first mention is made this is abbreviated in the text by omitting 'c.' or 'g.'.

The *NQO1* c.609 C>T SNP[3] (position according to GenBank Accession number J03934) was assessed by PCR-RFLP with an internal check on digestion. PCR was performed with the 'NQO1 cod-fw' and 'NQO1 cod-rv' primers (table 1), Super Taq DNA polymerase (HT Biotechnology, Sphaero Q, Gorinchem, the Netherlands), and annealing at 60°C. The resulting amplicon was digested with HinfI (Fermentas GmbH, St. Leon-Rot, Germany), which yielded fragments of 273, 119 and 23 base pairs (bp) for the C-allele, and fragments of 151, 122, 119 and 23 bp for the T-allele.

Table 1: *NQO1* and *NFE2L2* PCR and (pyro)sequencing primers

Name	Sequence (5′ → 3′)	Method	Measurement
NQO1 cod-fw	TGA GAA GCC CAG ACC AAC TT		
NQO1 cod-rv	GAA GGA AAT CCA GGC TAA GGA	PCR-RFPL	c.609C>T
NQO1 reg-fw	TTT TTC AGT ACA GAC GGG GCT TC		
NQO1 reg-rv	Biotin-GGC TGG TGT GGA GAT AGC AGT TA		
NQO1 pyrosequencing	GTG AGC CAC CGC GCC	Pyrosequencing	g.-718G>A
NFE2L2 fw	ATA CTG ACC ACT CTC CGA CCT AA		
NFE2L2 rv	Biotin-GTG GGG GGG GCT AAA GAT		
NFE2L2 pyrosequencing 1	CGT GGG AGT TCA GAG G	Pyrosequencing	g.-684G>A and g.-686A>G
NFE2L2 pyrosequencing 2	GAA CAC GAG CTG CCG		g.-650C>A

The *NQO1* regulatory SNP, g.-718G>A relative to the transcription initiation site (or -829 relative to the translation start) of GenBank Accession number M81596 and known under NCBI rs689457, was genotyped by pyrosequencing. We selected this SNP because it was found to have the highest frequency in our sequence survey of ~1100 bp of the NQO1 regulatory region upstream of the transcription initiation site among 96 individuals (unpublished results). PCR was performed with 'NQO1 reg-fw' and 'NQO1 reg-rv' primers (table 1), Super Taq DNA polymerase, and annealing at 61°C. The resulting 181 bp amplicon was used for a forward directed pyrosequencing analysis; primer 'NQO1 pyrosequencing' (table 1) was used to analyze the region -725 to -717 of *NQO1* in order to genotype the -718G>A polymorphism.

The *NFE2L2* SNPs reported by Yamamoto et al.[17], g.-650C>A, g.-684G>A and g.-686A>G relative to the transcription initiation site of GenBank accession number AC079305 (g.-733C>A, g.-767G>A and g.-769A>G, respectively, relative to the translation start of this accession number) were determined by pyrosequencing. PCR was performed with '*NFE2L2* fw' and '*NFE2L2* rv' primers (table 1), Super Taq DNA polymerase, and annealing at 58°C. The resulting 241 bp amplicon was used for two forward directed pyrosequencing analyses; primer '*NFE2L2*-pyrosequencing 1' (table 1) was used to analyze the region -686 to -677 of *NFE2L2* in order to genotype the -686A>G and -684G>A polymorphisms, and primer '*NFE2L2*-pyrosequencing 2' (table 1) was used to analyze the region -651 to -646 of *NFE2L2* in order to genotype the -650C>A polymorphism.

NQO1 and *NFE2L2* genotypes were in Hardy Weinberg Equilibrium among both controls and cases, as tested by a χ^2 test, with a possible exception for the *NQO1* -718 SNP, where χ^2 for cases was 3.04, p=0.081.

To test reproducibility, one row out of each DNA bank (8% of the population) was genotyped in duplicate; Four subjects could not be genotyped unambiguously for *NFE2L2*, the other measurements were 100% reproducible.

Statistical analysis. Fifteen individuals (11 cases, 4 controls) were excluded because of incomplete data on vegetable consumption. Twenty-four participants (17 cases, 7 controls) were excluded because blood was unavailable or the amount of DNA insufficient and therefore all genotyping results were absent. This resulted in a final study population of 1438 (740 cases and 698 controls). Additional individuals missing SNP data as a result of insufficient DNA varied from 1 (*NQO1* -718) to 17 (*NFE2L2* -684).

Haplotypes for *NQO1* and *NFE2L2* were estimated using the Hplus program version 2.5, available online (Fred Hutchinson Cancer Research Center. http://qge.fhcrc.org/hplus.) The two *NQO1* variants never occurred together on the

same allele, and neither did the three *NFE2L2* variants. In further statistical analysis, genotypes were therefore considered individually. Also, pairwise (and for power reasons not higher order) combinations were made. Case-control comparisons were made using conventional logistic regression. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated using the most common homozygous genotype as a reference group. Literature indicates that the *NQO1* 609 CT and TT genotypes respond differently biologically[6] and therefore they were not grouped together, despite low power for the TT genotypes. SNPs were analyzed in relation to adenoma risk in strata of other SNPs and strata of the following dietary and other lifestyle exposures that may influence NQO1: consumption of vegetables and fruits, meat (and subtypes), alcohol and coffee, and smoking. Smoking was defined as never or ever smoking. In addition, duration of smoking and number of cigarettes was evaluated, based on the (most equal) median split in controls. Consumption of fruits, vegetables, meat, alcohol (all in g/d) and coffee (freq/d) was divided into tertiles based on the distribution in the control group. For the analysis of higher order interactions, exposures were dichotomized, based on the median value of the control group. The reference group was the most common homozygous genotype in combination with low exposure. The p-value for interaction was calculated by χ^2 test comparing the –2 log likelihood (-2LL) values of the models with and without genotype-by-genotype or exposure-by-genotype interaction term(s). P-values lower than 0.05 were considered statistically significant. For reasons of power, the interaction analyses were restricted to individuals with the homozygous common and heterozygous genotypes, with the exception of the *NFE2L2* -686 SNP. Trend analysis was performed by substituting continuous values with the three control median values of the tertiles. Reported p-values are Wald χ^2 test p-values. Evaluated as possible confounders for smoking/dietary exposure-adenoma associations, which always included age (< and > 55 years) and sex (male/female), were: indication for endoscopy (bowel complaints, family history; yes/no), education (low/medium/high), smoking (never/ever; for dietary exposures), total energy intake (kJ/d), consumption of coffee (freq/d), alcohol, total meat, red meat, processed meat, total vegetables, cruciferous vegetables, total fruit and citrus fruit (all in g/d). Alcohol consumption affected the OR for number of cigarettes by more than 10% and was included in the smoking models and, for comparison, also in fruit and vegetable models.

Statistical analyses were performed using SAS software, version 9.1 (SAS Institute, Cary, NC).

To complement traditional analysis, we used a multifactor dimensionality reduction (MDR) approach[23]: a nonparametric method for detecting and characterizing nonlinear interactions. Two analyses were performed, using the MDR sofware available through www.epistasis.org (version 1.0.0): one containing all *NQO1* and *NFE2L2* SNPs and total fruit and vegetable consumption in tertiles, and one containing the additional attributes smoking (ever/never) and alcohol (median-split).

RESULTS

Characteristics of the study population are described in table 2, more elaborate descriptions can be found in previous reports[19, 24, 25]. Controls were younger, more often female and more often underwent endoscopy for reasons of bowel problems than cases. Cases were more often smokers and had a higher alcohol intake than controls.

Table 2: Selected population characteristics

Characteristic		Cases (n=*740*)	Controls (n=*698*)
General/medical		Median (p10-p90)	
Age	years	60 (44-72)	52 (33-69)
Sex	% female	46	62
Endoscopy indication	% bowel complaints[a, b]	47	77
	% ever[b]	67	56
	duration	30 (12-45)	22 (8-40)
Smoking	number/day	15 (4-25)	15 (5-25)
Dietary			
Total fruits & vegetables	g/day	277 (120-492)	260 (119-476)
total vegetables[c]	g/day	114 (69-180)	109 (68-169)
total fruits	g/day	127 (18-367)	125 (27-350)
Total meat	g/day	107 (40-178)	102 (35-172)
fresh red	g/day	60 (16-100)	53 (13-96)
processed	g/day	30 (6-75)	29 (5-73)
Alcohol	g/day	9 (0-42)	4 (0-31)
Coffee	freq/day	4 (2-7)	4 (0.4-8)
Energy	kJ/day	8378 (5885-11804)	8113 (5452-11808)

[a]bowel discomfort and defecation problems

[b]missing information on endoscopy indication: 9, missing information on ever/never smoking: 9

[c]does not include potato

The *NQO1* 609CT genotype was associated with a slightly higher colorectal adenoma risk (OR 1.27, 95% CI 1.00-1.62; table 3), and a similar observation was made for the *NFE2L2* -686AG genotype (OR 1.19, 95% CI 0.94-1.50; table 3), but not for the other SNPs.

Table 3: *NQO1* and *NFE2L2* genotyping results and adenoma risk

Genotype[a]		Cases (n=740)		Controls (n=698)		
		%	*n*	%	*n*	OR (95% CI)*
NQO1	CC	64.0	*472*	70.2	*490*	1 (ref)[b]
c.609 C>T	CT	32.6	*240*	26.5	*185*	1.27 (1.00-1.62)
	TT	3.4	*25*	3.3	*23*	1.03 (0.56-1.88)
NQO1	GG	79.0	*584*	78.2	*546*	1 (ref)
g.-718 G>A	GA	20.4	*151*	20.5	*143*	1.01 (0.77-1.32)
	AA	0.5	*4*	1.3	*9*	0.51 (0.15-1.77)
NFE2L2	CC	81.5	*596*	81.4	*566*	1 (ref)
g.-650 C>A	CA	17.5	*128*	17.8	*124*	1.01 (0.76-1.34)
	AA	1.0	*7*	0.7	*5*	1.19 (0.36-3.92)
NFE2L2	GG	75.5	*551*	77.4	*535*	1 (ref)
g.-684 G>A	GA	23.0	*168*	21.0	*145*	1.14 (0.88-1.46)
	AA	1.5	*11*	1.6	*11*	1.04 (0.43-2.50)
NFE2L2	AA	43.0	*314*	45.6	*316*	1 (ref)
g.-686 A>G	AG	45.4	*332*	42.1	*292*	1.19 (0.94-1.50)
	GG	11.6	*85*	12.3	*85*	0.99 (0.69-1.40)

*adjusted for age and sex

[a]missing genotype results *NQO1* c.609C>T: 3, g.-718A>G: 1, *NFE2L2* g.-650C>A: 12, g.-684G>A: 17, g.-686A>G: 14

[b]ref abbreviates reference category

There was a borderline significant interaction between the *NQO1* 609C>T and the *NFE2L2* -686A>G SNP (p for interaction=0.056). The higher adenoma risk associated with the *NQO1* 609CT genotype appeared to be absent in combination with the *NFE2L2* -686AA genotype (table 4). Risk was somewhat higher for the *NQO1* 609CT and *NFE2L2* -686AG genotype combination (OR 1.56, 95% 1.09-2.22; table 4) and, though with low power, was highest for the *NQO1* 609TT and *NFE2L2* -686GG genotype combination (OR 6.84, 95% CI 0.79-59.6; table 4). Results of haplotype analysis including the SNPs in both genes also pointed to a possible interaction between *NQO1* and *NFE2L2* (data not in table). The combination of the *NQO1* 609T and *NFE2L2* -686G variant nucleotides and wild-type nucleotides for the 3 other SNPs was statistically significantly associated with higher adenoma risk as compared to the combination of all five wild-type nucleotides: OR 1.72 (95% CI 1.18-2.49), with a frequency of 8.74% among cases and 5.10% among controls

compared with the wild-type combination of 31.6% and 31.5%, respectively. There was no interaction between the *NQO1* 609C>T SNP and the other SNPs in adenoma risk. The *NQO1* -718G>A SNP did interact with the *NFE2L2* -650C>A SNP (p for interaction=0.013), the combined heterozygotes showing an increased adenoma risk (OR 1.77, 95% CI 0.98-3.19; table 4) compared with the reference group of combined common homozygotes.

Table 4: Combined *NQO1* and *NFE2L2* polymorphisms and adenoma risk

NQO1 c.609 C>T and *NFE2L2* g.-686A>G		*NFE2L2* g.-686 A>G							
		AA		AG		GG			
		n[a] (*ca/co*)[b]	OR* (95% CI)	*n* (*ca/co*)	OR* (95% CI)	*n* (*ca/co*)	OR* (95% CI)	*p* int[b]
NQO1 c.609 C>T	CC	215/221	1 (ref)[b]	205/208	1.02 (0.77-1.35)	48/59	0.78 (0.50-1.20)	
	CT	90/82	1.00 (0.69-1.44)	117/76	1.56 (1.09-2.22)	31/25	1.22 (0.68-2.22)	
	TT	9/13	0.58 (0.23-1.43)	10/8	1.17 (0.44-3.13)	6/1	6.84 (0.79-59.6)	0.056
NQO1 g.-718 G>A and *NFE2L2* g.-650 C>A		*NFE2L2* g.-650 C>A							
		CC		CA		AA			
		n (*ca/co*)	OR* (95% CI)	*n* (*ca/co*)	OR* (95% CI)	*n* (*ca/co*)	OR* (95% CI)	*p* int	
NQO1 g718 G>A	GG	480/437	1 (ref)	91/103	0.84 (0.61-1.16)	5/4	1.06 (0.27-4.16)		
	GA	111/122	0.86 (0.64-1.16)	37/19	1.77 (0.98-3.19)	2/1	1.51 (0.13-17.3)	0.013[c]	
	AA	4/7	0.60 (0.16-2.18)	0/2	-	0/0	-		

*adjusted for age and sex

[a]numbers do not always add up due to missing values

[b]ca/co abbreviates cases/controls, ref abbreviates reference category, int abbreviates interaction

[c]omitting variant homozygotes due to low/absent cell numbers

Smoking (past or present) was associated with a higher adenoma risk (OR 1.43, 95% CI 1.13-1.80; table 5). There was a significant interaction between smoking and the *NQO1* 609C>T SNP (p for interaction=0.030, table 5); The increased adenoma risk with the *NQO1* 609CT genotype was clearly present among smokers (OR 1.96, 95% CI 1.40-2.76**),** but could not be seen among non-smokers (OR 0.91, 95% CI 0.62-1.35). Duration of smoking was positively associated with colorectal adenoma risk (not in table). Among *NQO1* 609CC genotypes the OR was 0.97 (95% CI 0.69-1.37) for less than 22 years and 1.51 (95% CI 1.09-2.09) for more than 22 years of smoking and among 609CT genotypes these risks were 1.65 (95% CI 1.01-2.71) and 2.16 (95% CI 1.45-3.21), respectively, both compared with non-smoking 609CC-genotypes. Adenoma risk did not increase with increasing number of cigarettes. There was no interaction between the *NQO1* -718G>A SNP and smoking.

Table 5: *NQO1* polymorphisms, smoking and adenoma risk

Smoking		Never		Ever		
		n[a] *(cases/controls)*	OR* (95% CI)	*n* *(cases/controls)*	OR* (95% CI)	*p* int[b]
All genotypes		*239/307*	1 (ref)[b]	*494/387*	1.43 (1.13-1.80)	
NQO1 c.609 C>T	CC	*163/211*	1 (ref)	*306/276*	1.24 (0.94-1.64)	
	CT	*67/88*	0.91 (0.62-1.35)	*172/96*	1.96 (1.40-2.76)	0.030
	TT	*9/8*	1.36 (0.50-3.72)	*16/15*	1.06 (0.49-2.30)	
NQO1 g.-718 G>A	GG	*191/234*	1 (ref)	*390/309*	1.36 (1.05-1.76)	
	GA	*48/69*	0.87 (0.57-1.34)	*102/73*	1.52 (1.05-2.22)	0.37
	AA	*2/4*	0.76 (0.12-4.65)	*2/5*	0.55 (0.10-3.00)	

*adjusted for age, sex and alcohol consumption (g/d)

[a]numbers do not always add up due to missing values

[b]ref abbreviates reference category, int abbreviates interaction

Following up on the literature, we also evaluated consumption of alcohol, coffee and (total, red and processed) meat for a possible interaction with the *NQO1* 609C>T SNP in colorectal adenoma risk. In this study, these factors did not interact significantly with the *NQO1* 609C>T SNP in colorectal adenoma risk. Alcohol consumption increased adenoma risk (data not shown in table) and this was additional to the effect of the *NQO1* 609C>T SNP: for the *NQO1* 609CC genotypes, the OR with the highest tertile of alcohol intake was 1.52 (95% CI 1.09-2.12) and for *NQO1* 609CT genotypes this was 1.82 (95% CI 1.19-2.80; p for interaction=0.70), both compared with the lowest tertile of intake and 609CC genotypes. Among smokers, the highest OR with the highest alcohol tertile was also observed among the 609CT genotypes: 1.98 (95% CI 1.08-3.65) versus 1.31 (95% CI 0.85-2. 40) for 609CC genotypes, compared with the lowest tertile of alcohol intake (data not in

table). However, among non-smokers, the highest OR with the highest tertile of alcohol intake was observed among the *NQO1* 609CC genotypes**:** 1.92 (95% CI 1.11-3.33, p for trend=0.025) versus 1.11 (95% CI 0.49-2.51) for 609CT genotypes, compared with the lowest tertile of alcohol intake. Interactions between the *NQO1* 609C>T SNP and alcohol consumption among non-smokers and smokers did not reach statistical significance: p for interaction=0**.**25 and 0.15, respectively.

High consumption of fruits and vegetables was associated with an increased adenoma risk: OR 1.36 (table 6). This was more associated with consumption of vegetables (OR for the highest vegetable tertile was 1.37, 95% CI 1.05-1.80), than with consumption of fruit (OR for the highest fruit tertile was 1.13, 95% CI 0.86-1.48; data not shown in table). There was no significant interaction between the *NQO1* 609C>T genotype or the *NFE2L2* genotypes and the consumption of fruits and vegetables, in adenoma risk (table 6). There was a weak suggestion for an interactive effect between the *NQO1* -718G>A SNP and consumption of fruits and vegetables (p for interaction=0.071): the highest tertile of fruit and vegetable consumption was associated with a higher risk among -718GG genotypes (OR 1.58, 95% CI 1.17-2.15) but not among -718GA genotypes (OR 1.05**,** 95% CI 0.67-1.66; table 6).

High fruit and vegetable consumption was also not associated with a lower adenoma risk for smokers. Compared to non-smokers with the common *NQO1* 609 variant (CC) and low fruit and vegetable intake (median-split), the highest adenoma risk was observed in smokers with the *NQO1* 609CT-genotype that had a high fruit and vegetable consumption (OR 3.01, 95% CI 1.78-5.10, data not in table). Our MDR analyses did not yield testing balance accuracies >0.55. With a cross validation consistency of 10/10, it did select the *NQO1* 609C>T and *NFE2L2* -686A>G SNPs, smoking and alcohol consumption as relevant variables.

Table 6: *NQO1* and *NFE2L2* polymorphisms, fruit and vegetable consumption and adenoma risk

Fruits&vegetables mean±sd (median)		T1[a]: <204 g/day 140±42 (143)		T2[a]: 204-335 g/day 264±38 (262)		T3[a]: >335 g/day 453±112 (421)			
		n[b] *(ca/co)*[c]	OR* (95% CI)	*n* *(ca/co)*	OR* (95% CI)	*n* *(ca/co)*	OR* (95% CI)	*p* trend	*p* int[c]
All genotypes		*216/233*	1 (ref)[c]	*251/233*	1.19 (0.91 -1.56)	*273/232*	1.36 (1.04-1.79)	0.0285	-
NQO1	CC	*136/156*	1 (ref)	*158/168*	1.12 (0.80-1.56)	*178/166*	1.32 (0.95-1.85)	0.095	
c.609 C>T	CT	*73/70*	1.17 (0.77-1.79)	*84/54*	1.71 (1.11-2.63)	*83/61*	1.64 (1.07-2.50)	0.23	0.64
	TT	*6/7*	0.82 (0.25-2.71)	*8/11*	0.83 (0.31-2.21)	*11/5*	2.38 (0.86-7.28)	0.17	
NQO1	GG	*169/194*	1 (ref)	*190/179*	1.25 (0.92-1.70)	*225/173*	1.58 (1.17-2.15)	0.0046	
g.-718 G>A	GA	*45/37*	1.40 (0.84-2.33)	*60/49*	1.43 (0.91-2.26)	*46/57*	1.05 (0.67-1.66)	0.43	0.071
	AA	*1/2*	0.74 (0.06-10.0)	*1/5*	0.27 (0.03-2.40)	*2/2*	1.82 (0.24-13.8)	0.41	
NFE2L2	CC	*170/190*	1 (ref)	*203/194*	1.20 (0.89-1.62)	*223/182*	1.44 (1.06-1.95)	0.024	
g.-650 C>A[d]	CA	*42/41*	1.16 (0.70-1.92)	*41/37*	1.23 (0.73-2.05)	*45/46*	1.24 (0.76-2.00)	0.73	
	AA	*1/2*	0.33 (0.03-4.29)	*4/0*	-	*2/3*	0.98 (0.16-6.12)	0.93	0.70
NFE2L2	GG	*160/177*	1 (ref)	*186/174*	1.19 (0.87-1.62)	*205/184*	1.31 (0.96-1.78)	0.13	
g.-684 G>A	GA	*49/48*	1.11 (0.69-1.78)	*58/53*	1.28 (0.82-2.00)	*61/44*	1.64 (1.03-2.62)	0.14	
	AA	*4/5*	0.78 (0.19-3.21)	*3/3*	0.97 (0.19-5.09)	*4/3*	2.29 (0.48-11.1)	0.079	0.86
NFE2L2	AA	*95/100*	1 (ref)	*114/106*	1.20 (0.80-1.80)	*105/110*	1.17 (0.78-1.77)	0.51	
g.-686 A>G	AG	*100/98*	1.21 (0.79-1.83)	*104/104*	1.19 (0.79-1.80)	*128/90*	1.72 (1.13-2.60)	0.055	
	GG	*18/33*	0.65 (0.33-1.27)	*29/21*	1.54 (0.80-2.98)	*38/31*	1.41 (0.79-2.53)	0.28	0.26

*adjusted for age, sex and alcohol consumption (g/d)

[a]tertiles of consumption, based on distribution in control group

[b]numbers do not always add up due to missing values

[c]ca/co abbreviates cases/controls, ref abbreviates reference category, int abbreviates interaction

[d]*NFE2L2* SNPs are shown because of the role of transcription factor NFE2L2 (Nrf2) in induction by fruits and vegetables

DISCUSSION

Our results confirm a role for the *NQO1* 609C>T SNP in colorectal tumor risk. The *NQO1* 609CT genotype, but not the 609TT genotype, was associated with a higher colorectal adenoma risk. The higher risk with the 609CT genotype was observed among smokers, but not among non-smokers. No such distinction was apparent for the 609TT genotypes. High consumption of fruits and vegetables did not protect those with the 609CT-genotype, but rather seemed to increase their risk further, especially among smokers. Interactions were observed between the *NQO1* 609C>T and the *NFE2L2* -686A>G SNPs, and between the *NQO1* -718A>G and *NFE2L2* -650A>C SNPs.

The *NQO1* 609CT genotype, but not the 609TT genotype, was associated with an increased colorectal adenoma risk. This finding is in line with a meta-analysis of the association between the *NQO1* 609C>T SNP and colorectal cancer[6], comprising 6 studies[26-31], which reported a significant slightly increased risk with the 609CT-genotype, but not the 609TT-genotype. It is also supported by more recent results for high risk colorectal adenomas from the UK flexible sigmoidoscopy screening trial[7]. As the C to T substitution results in loss of NQO1 enzyme activity[5], it is likely that the increased risk with the 609CT genotype relates to reduced detoxification. Lack of significance with the 609TT genotype may simply be due to the low prevalence of the 609TT genotype resulting in low power, though some studies of equal or smaller size do report an increased risk with the *NQO1* 609TT genotype (and not the CT genotype) in colorectal cancer[26, 32] and adenomas[8]. It may also relate to underlying differences in exposure to (pro)carcinogens metabolized by NQO1 between the study populations. Exposure to (pro)carcinogens is relevant for the amount of detoxification needed by the NQO1 enzyme, but also for its potential role as activator of (pro)carcinogens[13, 33].

Tobacco smoke is one such relevant environmental exposure[9, 10], being a source of the procarcinogen benzo(a)pyrene and its reactive benzo(a)pyrene quinone metabolites. NQO1 can specifically prevent formation of benzo(a)pyrene-quinone-DNA adducts[34]. We report an interaction between smoking and the *NQO1* 609C>T SNP in adenoma risk, the risk being highest with the 609CT genotype among smokers, but absent among non-smokers. There was no indication for an altered risk with the 609TT genotype. Our finding is supported by an earlier observation of interaction between smoking and the *NQO1* 609C>T SNP in distal colorectal cancer[28]. In colorectal adenoma risk, similar interactions were also observed before[7, 8], but these did not reach statistical significance. One of these adenoma studies did report a significant interaction when the *CYP1A1* 4889A>G SNP was included: the highest adenoma risk was observed among smokers with one or two

variants of both *NQO1* 609C>T and *CYP1A1* 4889A>G. Altogether, it is plausible that a mechanism associated with both smoking and the NQO1 enzyme adds to adenoma formation. In a separate study, we determined the *NQO1* 609C>T SNP and NQO1 enzymatic activity in rectal biopsies and also registered if subjects were current, ex or never smokers. Current smoking was associated with higher rectal enzymatic NQO1 activity than not smoking (144 and 96 nmol DCPIP/min/mg protein, respectively, p=0.0065) among *NQO1* 609CC-genotypes (n=59), but not among 609CT-genotypes (64 and 77 nmol DCPIP/min/mg protein, respectively, p=0.26; unpublished data). These data suggest that smokers with the 609CC-genotype induce their already higher NQO1 detoxification capacity and that smokers with the 609CT-genotype might be faced with a double burden: their already lower detoxification capacity and lack of induction. It is currently unclear how the absence of a higher adenoma risk with smoking among the 609TT-genotypes relates to this, as these subjects have no NQO1 detoxification capacity. Alternatively, they cannot bioactivate procarcinogens, such as nitrosamine in tobacco and this has been suggested as an explanation for a cancer-protective association with the 609TT genotype in Asians[6]. Another possibility is that total loss of activity is compensated by upregulation of other detoxification pathways, such as other quinone, carbonyl, and aldo-keto reductases[35].

Other relevant environmental exposures include consumption of alcohol and meat. A significant interaction has previously been reported between alcohol consumption and the *NQO1* 609C>T SNP, the 609CC genotypes having a higher adenoma risk with higher alcohol consumption[7], but we could not confirm this in our total population. Alcohol consumption did appear to increase adenoma risk among non-smoking 609CC-genotypes, but among smokers adenoma risk was still highest among 609CT-genotypes. This may indicate that the effect of smoking is stronger than the effect of alcohol consumption in our population, allowing the latter to become visible only in non-smokers. With respect to meat intake, an earlier study observed the highest colorectal cancer risk for high red meat intake among individuals with imputed intermediate NQO1 enzymatic activity (consisting of the *NQO1* 609C>T and 465C>T SNPs), and the lowest risk among those with deficient enzymatic activity[11]. We could not confirm an interaction between the *NQO1* 609C>T SNP and red meat consumption. Consumption of barbecued or grilled meat, as a source of PAHs, would also be interesting in this respect, but in this population, the frequency of consumption was too low to investigate, i.e. only ~12% of cases and of controls had barbecued or grilled meat once a month or more.

We hypothesized that consumption of fruits and vegetables protects the colorectum against the *NQO1* 609CT genotype and against the deleterious effects of quinone-type (pro)carcinogens such as present in cigarette smoke. This did not appear to be the case in our population. Higher consumption of fruits and vegetables even increased the adenoma risk among the NQO1 609CT genotypes, especially in combination with smoking. Mechanistically, this could be related to the action of an unspecified component that is widely present in fruits and vegetables. For example, a significant interaction between tobacco use and intake of β-carotene in colorectal adenoma risk has been reported[36], which consisted of a protective effect among non-smokers and an increased risk among ever smokers. In our study, there was no effect of β-carotene among non-smokers, but risk increased with the second and third tertile of β-carotene intake among smokers (data not shown). Interestingly, there was an increased adenoma risk with high fruit and vegetable consumption among *NQO1* -718 GG genotypes and this was absent among those with the -718 GA genotype. It is not known whether this represents a truly functional consequence, e.g. because the SNP is part of a regulatory sequence mediating the effect of a fruit and vegetable constituent.

It is also not yet known whether the SNPs in *NFE2L2* have consequences for the functioning of *NFE2L2*, but the significant gene-gene interactions between the *NFE2L2* and *NQO1* SNPs in this study indicate their possible functionality in (NQO1-related) adenoma tumor risk. Transcription factor NFE2L2 regulates transcription of several genes containing EpRE enhancers in their regulatory region. In light of that role, functional phenotypic differences between the NFE2L2 genotypes may have implications beyond the NQO1 enzyme and our findings need to be replicated in future studies including other enzymes.

Our study has strengths and weaknesses. The development of colorectal tumors is a complex process. More than one enzyme and more than one exposure play a role and we attempted to take this into account within the limits of our study size. We considered coding and regulatory region SNPs in *NQO1* and SNPs in the regulatory pathway of NQO1, as well as exposure to both proposed carcinogens and anticarcinogens. Exploration of three-way interactions with biological plausibility (such as smoking/fruits and vegetables/NQO1 SNP and smoking/alcohol/NQO1 SNP) can be seen as a strength, because this more closely represents real-life situations. However, as our study does not have enough power for three-way interactions, the analyses and their results should be interpreted as exploratory. Our genotyping assays were reproducible and the *NQO1* 609C>T genotype distributions in accordance with other northern European populations[27, 29, 37]. As far as we know, the distributions of the three *NFE2L2* SNPs have not been

reported for a (Northern) European population before. Our controls underwent endoscopy and this has the advantage of cases and controls being sampled from the same base population and of lower outcome misclassification because of verification of control status by endoscopy. Our positive association between fruit and vegetable consumption and adenomas requires further methodological consideration. In general, measurement of fruits and vegetables is difficult. Reproducibility of the FFQ was ~0.70 for vegetables and ~0.65 for fruits[38]. Validity relative to twelve 24hr recalls was ~0.35 for vegetables and ~0.60 for fruits[38]. These correlations are low, but no exception for FFQs[20]. They indicate that some degree of misclassification, especially for vegetables, may have occurred[39], but not to a greater extent than in other studies. Cases may have overreported their fruit and vegetable intake due to knowledge of adenoma status. Controls, being endoscopy-based and having a higher prevalence of bowel discomfort than cases, may have avoided intake of certain vegetables such as cabbage. We judge both types of bias unlikely, however, because adenoma patients do not receive dietary advice on fruits and vegetables in the Netherlands, earlier analyses restricted to new cases did not change conclusions[18] and only very few subjects have reported a change in vegetable intake due to bowel problems[18]. Bias may have been introduced due to non-response, but unfortunately we do not have sufficient data on patients not participating in the study to further evaluate a possible bias due to non-response or due to differences in outpatient clinic. Thus, we cannot fully rule out the possibility that selection bias, a problem often encountered in case-control studies, is one explanation why we see an increased adenoma risk with fruits and vegetables in this study. However, bias most likely is no explanation for differences in adenoma risk between genotypes.

In conclusion, smokers with the *NQO1* 609CT genotype have a higher colorectal adenoma risk and this is not counteracted by a high fruit and vegetable consumption. The regulatory *NQO1* SNP and the *NFE2L2* SNPs may also have modifying roles in colorectal tumor formation. As the *NQO1* 609C>T SNP demonstrates, it may not always be legitimate to group heterozygotes and homozygous variants together and genotypes should be placed in the context of polymorphisms in other genes and exposure to dietary and other lifestyle factors. This requires very large-scale epidemiologic studies. These studies, aided by human mechanistic and intervention studies, will need to confirm the role of the SNPs and also further evaluate the role of fruits and vegetables in this.

ACKNOWLEDGEMENTS

We thank Edine Tiemersma, Elly Monster, Maria van Vugt, Brenda Diergaarde, Petra Wark and Maureen van den Donk for their important roles in the study when they were at the Division of Human Nutrition, Wageningen University and Research Centre, the Netherlands; Elgin Lichtenauer for the development of the NQO1 genotyping assay; Jan Harryvan and Liesbeth Op den Camp for genotyping; Marga Ocke, from the National Institute of Public Health and the Environment, for providing the EPIC questionnaires and the subsequent nutrient calculations. We also thank the participants and the endoscopy staff of the following Dutch hospitals where the participants were recruited: Slingeland Ziekenhuis (Doetinchem), Ziekenhuis Gelderse Vallei (Ede), Radboud University Nijmegen Medical Centre (Nijmegen), Antonius Ziekenhuis (Nieuwegein), Meander Medisch Centrum (Amersfoort), Ziekenhuis Rijnstate (Arnhem), Ziekenhuis Rivierenland (Tiel), Slotervaart Ziekenhuis (Amsterdam), Jeroen Bosch ziekenhuis (Den Bosch), and Canisius-Wilhelmina Ziekenhuis (Nijmegen).

This work was supported by the Netherlands Organisation for Health Research and Development (ZonMW), grant number: 21000054, and the Dutch Digestive Diseases Foundation (MLDS), grant number WS 00-31.

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Preamble

The role of fruit and vegetable consumption and individual genetic variation in regulatory and coding DNA sequences of the glutatione S-transferase (GST) and NAD(P)H:quinone oxidoreductase (NQO1) detoxification enzymes was investigated in relation to GST and NQO1 enzyme level and activity, and in relation to colorectal adenoma risk.

In the previous chapters, the background and rationale of the studies were described, and the methods and results were discussed by detoxification system (GSTs and NQO1) and endpoint (enzyme phenotype and colorectal adenomas).

In this chapter, the findings are integrated, placed in a broader context of concepts and methodology, and their implications are discussed. The focus of the chapter will be on the concept of polymorphism functionality, interpreted from and structured into a molecular, physiological and epidemiological perspective, with emphasis on the latter perspective. Finally, a societal perspective is reflected upon, and some suggestions for future research are made. The chapter starts with a brief recapitulation of the main findings.

Chapter 6

General Discussion

MAIN FINDINGS

The main findings are summarized by detoxification system and by endpoint, below and in table 1. It is based mostly on the polymorphisms for which some knowledge concerning their functionality was already available. An elaborate summary can be found in addendum I.

In general, regulatory SNPs in *GSTA1*, *GSTP1* and *NQO1* with convincing differences in their responsiveness to fruit and vegetable intake were not found.

GSTs: rectal enzyme phenotype. The most important determinant of rectal GST activity was the *GSTP1* 313A>G polymorphism, GST activity being lower with the 313 G variant. Overall, fruit and vegetable consumption was not associated with higher GST activity, but individual botanicals were associated with higher or lower levels of individual GST enzyme levels (e.g. Alliaceae and GSTP1 and Apiaceae and GSTM2, respectively). The correlation between rectal and white blood cell GST activities was low.

GSTs: colorectal adenomas. High consumption of cruciferous vegetables appeared to increase adenoma risk among those with slower metabolizing GST variants, especially the combination of the *GSTA1* -69 T and *GSTP1* 313 G variants, contrary to our hypothesis. The *GSTM1* and *GSTT1* genotypes, and cruciferous vegetable intake did not seem to interact with respect to colorectal adenomas.

NQO1: rectal enzyme phenotype. The most important determinant of rectal NQO1 activity was the *NQO1* 609C>T polymorphism, NQO1 activity being lower with the 609 T variant. Overall, fruit and vegetable consumption was not associated with higher NQO1 activity, but rather with lower values, especially for the botanical subtype Apiaceae. *NQO1* mRNA and NQO1 activity correlated among NQO1 609CC genotypes but not among CT genotypes. Rectal and white blood cell NQO1 activities did not correlate.

NQO1: colorectal adenomas. The *NQO1* 609CT genotype was associated with an increased adenoma risk among smokers. Consumption of fruits and vegetables did not decrease this risk. *NQO1* and *NFE2L2* genotypes interacted in colorectal adenoma risk.

Table 1: Main findings, by detoxification enzyme and by endpoint

Endpoint	Determinant[a]	Association with endpoint	Chapter
Glutathione-S transferases (GSTs)			
Rectal total GST activity	*GSTP1* c.313A>G SNP	↓ and ↓↓ for 313AG and 313GG genotypes compared to 313AA	2, table 4
	GSTA1 g.-69C>T SNP	↑ for -69CT and -69TT genotypes compared to -69CC	
	Fruit or vegetable consumption	↔ for yes compared to no consumption (except fruit and GSTM1 null)	2, table 5 + text
Rectal GSTM1 level	Citrus fruit + juice consumption	↑ for yes compared to no consumption	2, table 3
	Cruciferae consumption	Not sign: ↑ for yes to no consumption	2, table 3
Rectal GSTM2 level	Total vegetable consumption	↓ for yes compared to no consumption	2, table 3
	Apiaceae consumption	↓ for yes compared to no consumption	
Rectal GSTT1 level	Alliaceae consumption	↑ for yes compared to no consumption	2, table 3
	Cruciferae consumption	Not sign: ↑ for yes to no consumption	2, table 3
Rectal GSTA level	*GSTA1* g.-69C>T SNP	↓ and ↓↓ for -69CT and -69TT genotypes compared to -69CC	2, figure 1
Rectal GSTP1 level	*GSTP1* c.313A>G SNP	↑ and ↑↑ for 313AG and 313GG genotypes compared to 313AA	2, text
	Alliaceae consumption	↑ for yes compared to no consumption	2, table 3
	Cucurbitaceae consumption	↑ for yes compared to no consumption	2, table 3
Colorectal adenoma risk	Cruciferae consumption and *GSTP1* c.313A>G SNP	↑ for high compared to low consumption among 313GG	3, table 2
	Above and *GSTA1* g.-69C>T SNP	↑ for high compared to low consumption among 313GG or -69TT	3, table 2
NAD(P)H quinone:oxidoreductase (NQO1)			
Rectal NQO1 enzymatic activity	*NQO1* c.609C>T SNP	↓ for 609CT genotype compared to 609CC genotype	4, figure 1 & table 3
	Fruit or vegetable consumption	↔/↓ for yes compared to no consumption	4, table 2 & 5
	Apiaceae consumption	↓ for yes compared to no consumption	4, table 2
Colorectal adenoma risk	*NQO1* c.609C>T and *NFE2L2* g.-686A>G	↑ for *NQO1* 609CT/*NFE2L2* -686AG and 609TT/-686GG combined[b]	5, table 4
	NQO1 g.-718G>A and *NFE2L2* g.-650C>A	↑ for *NQO1* -718GA and *NFE2L2* -650CA combined[b]	5, table 4
	NQO1 c.609C>T and smoking	↑ for *NQO1* 609CT and ever smoking[c]	5, table 5
	NQO1 c.609C>T, smoking and total fruit and vegetable consumption	↑ for *NQO1* 609CT and ever smoking and high fruit and vegetable consumption[c]	5, text

[a]no data on GSTP1 g.217G>A, GSTP1 g.227G>A and GSTP1 g.272C>G SNPs in adenoma study.

[b]compared to both common variants

[c]compared to no smoking/low consumption and most common variant

POLYMORPHISM FUNCTIONALITY IN RELATION TO FRUIT AND VEGETABLE CONSUMPTION AND DETOXIFICATION: SUBJECT MATTER AND METHODOLOGICAL CONSIDERATIONS FROM DIFFERENT PERSPECTIVES

Here, the findings are discussed in light of the concept of polymorphism functionality, from a molecular, physiological, epidemiological and societal perspective. These perspectives represent and structure a continuum of integration levels of increasing complexity, through which the functionality of a polymorphism can persist. Functionality is broadly defined as the genetic difference having any consequence or not. An effect that persists through the higher integration levels is considered most relevant. The molecular, physiological and epidemiological perspectives start with a brief introduction and the position of relevant research questions. These are discussed with respect to content under the heading 'Subject Matter'. Each perspective is bound by its available methodology, and this is accounted for under the heading 'Methodology'. Subsequently, the societal perspective is addressed.

The physiological and epidemiological perspectives are the core of this thesis, and the molecular and societal perspectives are touched upon to complement the continuum of integration levels on both sides.

MOLECULAR PERSPECTIVE

Definition. The molecular perspective is considered to include a comparison between different genotypes with respect to the organization of the DNA, the regulation of DNA transcription and environmental influences on this, and the biochemical properties of the proteins that are produced.

Concept. In regulatory regions, a distinction can be made between genetic differences being functional in the absence or presence of environmental stimuli. On the one hand, genetic variation can have constitutive consequences, i.e. a basal difference in transcription rate. On the other hand, genetic variation can have consequences in response to environmental stimuli, e.g. a different induction effect. In our studies of colorectal cancer prevention, the main interest was in the latter mechanism, i.e. gene-fruit & vegetable interactions.

Candidate genes. *GSTA1*, *GSTP1* and *NQO1* were selected as candidate genes, because they code for common biotransformation enzymes which metabolize carcinogens relevant for the colorectal area and because they have been reported to

be inducible in humans by consumption of fruits and vegetables (as explained in the Introduction).

Study questions. One can ask the following questions fitting within the molecular perspective with regard to the *GSTA1, GSTP1* and *NQO1* genes:

Q1) Have genetic variants been identified in the regulatory region?
Q2) If so, do they confer different phytochemical-induced transcription levels?
Q3) If so, what is the combined effect of the identified regulatory region polymorphisms and known coding region polymorphisms on phenotype?
Q4) Do the known coding region genetic variants encode biotransformation enzymes which metabolize phytochemicals differently?

MOLECULAR PERSPECTIVE: SUBJECT MATTER

The search for polymorphisms in regulatory regions and the mechanistic assessment of their functionality was the subject of a complementary study (A.M.J.F. Boerboom, J.M.M.J.G. Aarts and I.M.C.M. Rietjens).

Regulatory SNPs (Q1). The SNPs to be investigated further in relation to enzyme phenotype and adenoma occurrence ideally have mechanistic back-up of their functionality and occur with a frequency that would give these studies sufficient statistical power (the latter depends on many things, but a minimum requirement for the SNPs could be that they are 'common', i.e. with a minor allele frequency of more than 10%[1]). Only few polymorphisms were idenfied to occur at a frequency suitable for further studies. Of these, only one was in a regulatory region (*NQO1*) and three were in an intronic region (*GSTP1*). No SNPs were observed in the *NQO1* and *GSTP1* electrophile responsive elements (EpREs; *GSTA1* is not known to contain an EpRE). Recently, SNPs in EpREs were reported to occur in other genes, but not in *NQO1* and *GSTP1*[2]. In the literature, functional haplotypes in the regulatory region have been reported in the *GSTA1* gene[3] and in the *GSTP1* gene[4]. The haplotype in *GSTA1* could be confirmed, the haplotype in *GSTP1* could not.

Functionality of regulatory SNPs (Q2 and 3). The studies concerning the plant component responsiveness or basal functionality of the polymorphisms were still on-going at the time this thesis was written and thus mechanistic back-up cannot be established at the time. A stable Hepa-1c1c7 luciferase reporter cell line containing the human *NQO1* electrophile responsive element (EpRE) was developed[5]. This assay can be used to screen dietary constituents and to better

understand mechanistically their EpRE mediated gene transcription activation. The transfected construct can also be modulated to contain *NQO1* promoter polymorphisms and can consequently be used to experimentally test differences in transcription activation between individual genotypes as well as between dietary constituents[6].

The *GSTA1* regulatory region haplotype has been reported to have a different basal transcription rate[6], as described in the Introduction.

Functionality of coding SNPs (Q4). From the scientific literature, some GSTP1 and NQO1 polymorphisms in coding regions, were already known. Their mechanistic functionality, i.e. a different substrate specificity for variants of *GSTP1*[7, 8] and *NQO1*[9, 10], has been described in the Introduction. With respect to *GSTP1*, there is not only a difference in substrate specificity for carcinogenic substrates, but also for supposed anticarcinogenic substrates such as glucosinolate-derivatives[11-13] The known functional coding variants were included for evaluation in our genotype-phenotype and case-control study.

Future. Ideally, a number of (frequently occurring) regulatory SNPs would have been identified, their molecular functionality would have been known, and they would have been related to phenotypic outcomes on higher integration levels. For an example of such a multidisciplinary approach, see ref[14]. Unfortunately, this did not work out as such for our multidisciplinary project. The multidisciplinary approach is still deemed highly valuable, however. For the future, one improvement to the approach may be to allow for a (greater) time lag between the molecular work and the work on the higher integration levels.

MOLECULAR PERSPECTIVE: METHODOLOGY

Screening for promoter SNPs. Regions of the *GSTA1*, *GSTP1* and *NQO1* promoters of 96 individuals (from the same base population as in subsequent studies) were sequenced. This number should suffice to identify SNPs occurring at frequencies relevant for further studies. It did not yield many frequently occurring SNPs, however. For the relevant DNA sequences and observed polymorphisms, see addendum II. There are a number of possibilities why other functional polymorphisms in regulatory regions were not found. If SNPs with regulatory potential exist, they may be outside the sequenced region, do not occur in our population or are missed in sequencing. Also, a gene may be affected by polymorphisms in transcription factors regulating it.

Sequencing scope. Regulatory region SNPs could have been missed because sequencing did not cover a sufficiently large area. For *NQO1*, an upstream region of ~1100 bp from the transcription starting point was covered. In the *NQO1* gene,

functional genetic variation within regulatory sequences has been suspected because of observed differences in transcription level[15]. However, lack of induction was reported not to be due to regulatory region genetic variation when ~2000 bp of *NQO1* regulatory region from 6 cell lines were sequenced[16]. Based on the above, it is possible that common, functional SNPs are not present in the regulatory region, but more research is warranted.

Functional SNPs could be present at large distance from the transcription starting point. The gene control region generally comprises several transcription factor binding sites that may influence (activate, repress) gene transcription and these could also be subject to the influence of fruit and vegetable components.

Also, other steps in the regulatory process can be influenced by genetic variation. The SNP at position +465 relative to the transcription starting site in the *NQO1* gene is a good example of a functional SNP in an intronic sequence. It leads to alternative splicing, with consequences for the quinone binding site[17]. This particular polymorphism is infrequent, however, and thus not suitable for epidemiologic studies unless they are extremely large.

Population. With regard to *GSTP1*, amplification of relevant *GSTP1* promoter sequences by PCR repeatedly failed due to GC-rich areas and the promoter area to be sequenced was downsized. Eventually, a ~900 bp region was sequenced, reaching into the regulatory region to position -405 (acknowledgement Dr. M. Visker, dept. of Human Nutrition). Cauchi et al. reported a regulatory haplotype responsive to phytochemicals *ex vivo* after our data collection period was over, i.e. one homozygous and three heterozygous genotypes among 40 Caucasians[4]. Four of their SNPs, starting at position -282 relative to the transcription initiation site, were within the region that was sequenced in our population (for the relevant DNA sequences and observed polymorphisms, see addendum II). It is unclear why they were missed.

Transcription factors. Variation may also occur in other, pathway-related genes. The most important regulatory factor for EpRE-related transcription, NFE2L2, has been reported to be polymorphic: three SNPs and a deletion polymorphism were found[18]. The SNPs were included in our investigations. Molecular studies investigating their functionality are lacking.

MOLECULAR PERSPECTIVE: CONCLUSION

At this point, it remains uncertain whether the *GSTA1*, *GSTP1* and *NQO1* genes contain regulatory polymorphisms with experimentally shown functional differences in relation to inducing fruit and vegetable components. New results are expected in this field in the near future.

PHYSIOLOGICAL PERSPECTIVE

Definition. The physiological perspective is considered to include a comparison of enzyme levels and activities (phenotype) in tissues of individuals with different genotypes, and dietary and other environmental factors influencing this enzyme phenotype.

Concept. Enzyme phenotyping can yield valuable information on biotransformation capacity and regulation of human carcinogen-metabolising enzymes, although it is labour-intensive and costly. The rationale behind measuring it is that the enzymes lower the levels of carcinogens in the tissues and cells by inactivating carcinogenic substances[19]. It gives more information on the actual level of protection compared to the molecular perspective. This is especially true when phenotyping is performed in the actual tissue of interest. When genotype-phenotype relations are established, it can also provide the rationale for using polymorphisms, as markers which are assessed relatively quickly and inexpensively, in epidemiological studies.

SNP selection. SNPs were selected based on our own sequencing results (*GSTP1* 217G>A, 227G>A, 272C>G , *NQO1* -718G>A SNP), based on molecular and phenotypic knowledge from the literature (*GSTA1*-69C>T, *GSTP1* 313A>G and *NQO1* 609C>T), and based on literature frequency reports (*NFE2L2* -650C>A, -684G>A, -686A>G SNPs).

Study questions. The following questions fitting within the physiological perspective were asked with regard to GSTA1, GSTP1 and NQO1 phenotype:

Q1) Do the genetic variants result in different protein or mRNA levels in rectum?

Q2) Do the genetic variants result in different protein activity in rectum?

Q3) Does consumption of fruits and vegetables, or their subtypes, result in different levels or activity of GSTA1, GSTP1 or NQO1 in rectum?

Q4) Does an interaction exist between fruit and vegetable consumption and GSTA1, GSTP1 or NQO1 genetic variants in GSTA1, GSTP1 or NQO1 protein levels or activity in rectum?

Q5) Does GST or NQO1 phenotype in blood reflect phenotype in rectum?

PHYSIOLOGICAL PERSPECTIVE: SUBJECT MATTER

Genotype – enzyme phenotype (Q1 and 2). *SNPs with known functionality.* The best-known polymorphisms in *GSTA1*, *GSTP1* and *NQO1* (*GSTA1*-69C>T, *GSTP1* 313A>G and *NQO1* 609C>T) were clearly associated with a difference in enzyme

phenotype in the rectum, as had been described during and before our work, in different tissues (*GSTA1*:[3, 20]; *GSTP1*:[21]; *NQO1*: [22-25]). For *GSTA1*, the difference was in the level of protein. For *NQO1* and *GSTP1*, the difference was in enzymatic activity. For the latter, this was reflected in the total GST activity. GSTP1 protein level and *NQO1* mRNA were measured to be able to determine a transcription effect, but the polymorphisms affecting the enzymatic functioning of the enzyme also seemed to affect transcription, in the opposite direction. Both the *GSTP1* 313A>G and *NQO1* 609C>T polymorphisms resulted in lower activity, but their expression levels seemed to be higher. This shows that mRNA and protein levels bear a complicated relation to ultimate physiological functionality, i.e. the actual activity of the enzyme.

SNPs with unknown functionality. The presence of the relatively strong differences between the *GSTP1* 313 and *NQO1* 609 genotypes makes detection of a difference on the enzyme level for the other polymorphisms more difficult. Their analysis can be stratified for the *GSTP1* 313 and *NQO1* 609 genotypes, but this reduces power. The *GSTP1* intronic polymorphisms were not convincingly associated with GSTP1 level or total GST activity (chapter 2). The same applies to the *NQO1* promoter polymorphism and NQO1 phenotype (chapter 4). The *NFE2L2* promoter polymorphisms may have some relation with NQO1 phenotype. The *NFE2L2* -684G>A polymorphism was associated with higher NQO1 mRNA level, the difference between the *NFE2L2* genotypes being most pronounced among those with the common homozygous *NQO1* 609CC genotype. The associations between the *NFE2L2* polymorphisms and GST phenotypes were also evaluated. As the results have not been submitted to a scientific journal, they are included in addendum III. In short, the *NFE2L2* -684GA genotype was associated with a higher GSTM1 level and total lymphocyte GST activity. The *NFE2L2* polymorphisms did not significantly affect total rectal GST activity. So far, other studies investigating their physiological functionality have been limited to an observational study which concluded that the SNPs are associated with cellular indicators of gastric mucosal inflammation[26], which shows the extensive role of NFE2L2 as a transcription factor.

Fruits & vegetables – enzyme phenotype (Q3). Fruit and vegetable subtypes may contain components with opposing influences on colorectal enzyme phenotype.

Induction. Allium consumption was associated with *increased* rectal GST protein levels, e.g. the level of GSTP1 and GSTT1. Thus, it may be relevant in colorectal cancer prevention[27]. For GSTP1 there is mechanistic support for an induction effect by Allium vegetables[28]. Crucifers did not increase enzyme levels or activity, at least not statistically significantly. Several studies, however, have

shown higher GST capacity with higher crucifer intake[29-31] [32-36]. Possibly here, *type* of crucifer also matters. Above-mentioned studies were performed with Brussels sprouts, broccoli and red cabbage. In the studies by Steinkellner and co-workers, there was no induction by broccoli and white cabbage[37]. In general, cauliflower is the main crucifer of consumption in the Netherlands, as a toplist of most frequently consumed vegetables in the 1998 Dutch National Food Consumption Survey showed (chapter 3). In our genotype-phenotype study, the order of highest to lowest crucifer consumption was cauliflower, white cabbage, Brussels sprouts, broccoli, traditional 'potatoe-and-crucifer' hodgepodge, pointed cabbage and radish (data not shown). In addition, *dose* and *duration* of exposure may also be associated with different mechanisms. Feedback mechanisms may be invoked. For example, in the Oltipraz trials, induction of GST and NQO activity was observed after a single dose, but the effect was lost after multiple doses[38, 39].

Repression. Total fruit and vegetable consumption was associated with *lower* total GST activity and GSTP1 level and total vegetables with lower *NQO1* mRNA. Compositae consumption was associated with lower *NQO1* mRNA and Apiaceae consumption with lower NQO1 enzymatic activity and lower GSTM2 level. Gene suppression by fruits and vegetables is biologically plausible. Persson et al. observed GSTP1 downregulation after three weeks of total vegetable intervention and speculated that this was compensatory, the result of increased external supply of antioxidants. An intervention study with polyphenol-rich fruit juices also showed GSTP1 protein downregulation, in leukocytes[40]. In vitro studies by Van Zanden[41] en van Haaften[42, 43] show that GSTP1 can be inhibited by the flavonoid quercetine and by tocopherols. Pro-vitamin A carotenoids may also have a repressive effect on both GSTP1 and NQO1 by the retinoic acid (RA)-AP-1 mechanism discussed in chapter 4. Certain coumarins and flavones are competitive inhibitors of NQO1 activity that compete with NAD(P)H for binding to NQO1[44, 45] [44-48], resulting in lower activity. However, the latter type of inhibition could not be measured in our study (see Methods), and thus inhibition of enzymatic activity is therefore no explanation for our observation. It may nonetheless play a role in vivo. For example, a human intervention study found Apiaceae to inhibit CYP1A2 activity, measured indirectly as urinary caffeine metabolite ratios[49]. Peterson et al. subsequently identified constituents in Apiaceae, certain furanocoumarins and flavonoids, likely to be responsible[50].

Genotype – fruits & vegetables interaction in enzyme phenotype (Q4). Some examples were seen of fruit and vegetable consumption being associated with enzyme phenotype which appeared to differ among genotypes (chapter 2 and 4, see also Add I). However, our genotype-phenotype study remains exploratory in

this respect. For appropriate power, complex interactions involving several polymorphisms and several environmental influences require sample sizes that are hard to accomplish in enzyme phenotyping studies.

Enzyme phenotype in rectum and white blood cells (Q5). The major polymorphisms for total GST activity and NQO1 activity in the rectum, the *GSTP1* 313A>G and the *NQO1* 609C>T SNP, did not so much affect white blood cell total GST activity and NQO1 activity. This is likely the result of tissue specific expression. Different GSTs are expressed in different quantities in different tissues[51] (resulting in tissue-specific susceptibility to genotoxicity). In addition, clear differences in GST expression have been found between different lineages of hematopoietic cells[52] (which may the defence capability of cells from those lineages). In lymphocytes, as in rectum, GSTP1 is reported to be the most abundant isoenzyme[52, 53]. This probably explains why the difference in GST activity between the *GSTP1* 313A>G genotypes could also be seen in lymphocytes. GSTM1 is also thought to be important in lymphoid cells[52]. These polymorphisms were not seen to influence lymphocytes, but we did see an effect on leukocytes, which consist of ~30% lymphoid cells. NQO1 is not highly expressed in blood cells. Besides genetic differences, tissues are supplied differently with diet-derived compounds. Blood is thus not a good surrogate tissue for rectal tissue and invasive studies taking biopsies remain necessary. An alternate explanation for lack of association between blood (both lymphocytes and leukocytes) and rectal tissue is that they represent different terms of exposure (short-term vs long-term). This would still mean they are not interchangeable (but could be complementary, depending on the research question to be answered).

PHYSIOLOGICAL PERSPECTIVE: METHODOLOGY

Assessment of enzyme phenotype. Enzyme levels are the result of many biological factors. Furthermore, the reproducibility and validity of the chosen phenotypic measurements need to be considered. Issues influencing internal and external validity discussed here are the use of specific chemicals, detoxification vs bioactivation (or: the paradigm that higher is better) the depth and localisation of rectal biopsy and reversible inhibition.

Specific chemicals. Enzyme activity is not an absolute characteristic of a certain amount of a certain protein, but is relative to the substrate that is used. In the case of total GST activity, which consists of the sum of the activities of the GST subtypes, it is also determined by the relative substrate specificities of the contributing GST isoenzymes and the relative amounts in which they occur. The latter is tissue specific. Illustrating this, is a controlled feeding trial, where the

vegetable diets did not have an effect on GST activity in serum measured with CDNB as a substrate, but did affect GST activity measured with NBD-Cl as a substrate[54]. CDNB is a well-known generic substrate and was used in our studies for this reason. However, it may not best represent enzymatic activity towards, and thus protection against, all individual relevant carcinogens. Two examples by which this may affect our studies and other studies: GSTT1 has no metabolic activity for CDNB and GSTA1 relatively low activity. Firstly, if a substrate had been chosen to measure (total) GST activity that also allowed detection of GSTT1 activity, then maybe a protective effect of Allium vegetables on total GST activity would have been shown in chapter 2, as opposed to only on GSTP1 and GSTT1 protein levels. Secondly, GSTA1 is the only GST with high specificity for N-acetoxy PhIP, a dietary carcinogen that is very relevant for humans[55] and CDNB will say relatively little about protection against this carcinogen. Using several substrates in measuring enzyme activity may thus yield valuable information. Despite substrate issues, enzymatic activity probably does represent a more relevant measure with regard to protection against carcinogens than enzyme levels.

Enzyme activities and levels are commonly expressed by the amount of total protein to allow comparison between study subjects. As the methods to determine total protein are based on different chemical principles[56-58], comparison between studies is not always possible. A different issue with respect to normalising to total protein is the possibility that other proteins are also affected by induction[53]. This will affect total protein, and thus the absolute degree of change in GST or NQO1 level or activity may be attenuated by an accompanying change in total protein. For leukocytes, a solution is to measure by 10^6 cells instead of total protein[40].

Detoxification versus bioactivation. Measurement of phase II enzymes addresses only one side of the medal. For detoxification purposes it may be important to know if fruits and vegetables also affect phase I enzymes, which may activate (pro)carcinogens, and if polymorphisms exist in phase I enzymes that affect their capacity. Also, (the level of) exposure to (pro)carcinogens is important, and we attempted to take this into account in our case-control comparison by subgroup-analyses. Effects of (pro)carcinogen metabolism can also be measured and may give more insight in the functionality of the polymorphism. An example of this is the study by Hecht et al.[59], in which vegetables were fed and in which (pro)carcinogen exposure (smoking) and a measure of consequence (NNK metabolites in urine) were taken into account.

Depth and localisation of biopsy. Rectal biopsies may have contained not just epithelial tissue, but also underlying connective tissue and the ratio between the two tissue types may have varied between individuals due to random or

endoscopist-specific differences in biopsy depth. This is relevant, because NQO1 is not found in connective tissue. Sole presence of epithelial tissue could be checked under light microscopy, but this would preclude snap-freezing of samples and thus possibly affect enzyme activity. The ratio of mucus secreting goblet to absorptive cells increases toward the rectum. NQO1 expression is low in goblet cells compared to the absorptive cells. As biopsies were taken over a small distance of 10 cm, biopsy localisation probably did not affect comparability in our study, but may affect generalisability to the colon. The same is true for GSTs, their levels are also known to decrease towards the rectum.

Reversible inhibition. A general drawback of measuring enzyme phenotype is that reversible inhibition (e.g. competition with NAD(P)H for binding to NQO1) cannot be measured in a cell lysate /*ex vivo*, because the inhibitory dietary compound will already have diffused away from the (NQO1) enzyme. However, it may still be relevant as a mechanism and have consequences for (NQO1) capacity. For some enzyme systems, it is possible to measure the consequences of inhibition somewhere further in the pathway, illustrated by the study measuring urinary cafeine metabolite ratios in CYP1A2 metabolism[49].

Assessment of fruit and vegetable consumption by food record. In the genotype-phenotype study, fruit and vegetable consumption was measured by food record. A food record is thought not to rely on memory and to be a specific reflection of actual intake[60]. Information on actual, and not habitual, consumption was desired in relation to enzyme phenotype for biological reasons, i.e. the pathway from consumption to expression and translation takes hours to a few days, known from cell studies and shown in human intervention studies[29, 38]. As not every fruit and vegetable subtype (for an overview of the botanicals and the individual subtypes that belong to the classes, see Addendum IV) is eaten by all study subjects within the days before endoscopy, exposed versus non-exposed groups can be formed. Two groups (yes and no consumption) were formed for practical reasons of power. Also it was expected that the difference of effect between no and any exposure is greater than between different levels of exposure. This may not be true for all vegetable subtypes or thresholds may exist at high doses. For example, an association suggesting an induction effect of cruciferous vegetables could not be demonstrated. Possibly, high doses such as used in controlled feeding trials are needed or an effect is confined to only some cruciferous vegetables and not others that are commonly eaten. Furthermore, a classification into botanically defined subtypes is only useful to some extent. In the context of this study, the classification should be able to show effects of phytochemicals that are mainly confined to these subtypes, but compounds that

are more widespread will need a different grouping or estimation from the total amount of fruits and vegetables that are consumed. Of course, intervention trials can give better answers with respect to underlying mechanisms and causality. In order to evaluate whether fruits and vegetables truly enhance the human detoxification system in vivo, a range of trials would have to be designed taking into account at least: different types of vegetables, doses, exposure times, phase II enzymes, phase I enzymes, polymorphisms, tissues and base line enzyme values. This is a true challenge. Also, studies would be needed to see under which circumstances increased detoxification capacity actually protects against cancer.

Population. In the genotype-phenotype study, subjects came from a population that was referred to an endoscopy clinic. The exclusion criteria were intended to secure a study group with relatively normal mucosa. Yet there is still a chance that those with seemingly normal mucosa had an unmeasured underlying defect related to GST and NQO1 enzymes which affected phenotype as compared to those from the general population. Genotype distributions were comparable to other populations, which can be interpreted as an indication that a normal fraction of the population was sampled with respect to GST and NQO1, although this is difficult to say in a population this size. There were no individuals with the low frequency *NQO1* 609TT genotype. This could be due to the relatively small sample size, may mean that homozygosity is related to the exclusion criteria, or it could even be the result of cross-contamination during DNA isolation.

PHYSIOLOGICAL PERSPECTIVE: CONCLUSION

GSTA1, *GSTP1* and *NQO1* contain polymorphisms with consequences for enzyme phenotype. Different botanicals may be capable of upregulating or downregulating enzyme phenotype. The effect of polymorphisms and fruit and vegetable consumption may be interactive, but this was not clearly shown. Measurements should be performed in the colorectum, white blood cells are not a good surrogate tissue.

EPIDEMIOLOGICAL PERSPECTIVE

Definition. The epidemiological perspective is considered to include a comparison of disease occurrence or intermediate clinical disease markers between groups of individuals with different genotypes, and dietary and other lifestyle factors influencing this.

Concept. The conceptual framework of this perspective is that genetic susceptibility only matters in the presence of relevant environmental stimuli, i.e.

exposure to a relevant amount of carcinogens is needed for a genetic variant with lower detoxification potential to take effect and similarly, exposure to a relevant amount of anti-carcinogens is needed for a genetic variant with a higher induction potential of detoxification enzymes to take effect. Or in other words, from a consequential perspective: without exposure to carcinogens, there is no (visible) difference in toxicity; without exposure to anticarcinogens, there is no (visible) difference in protection capacity.

SNP selection. In the epidemiological studies, the same SNPs were evaluated as were in the physiological studies (see p 7), with the exception of the *GSTP1* intronic SNPs.

Study questions. The following questions fitting within the epidemiological perspective were asked with regard to an intermediate disease marker in the colorectal cancer pathway, adenomas:

Q1) Is there an association between the polymorphisms and the occurrence of adenomas?

Q2) Is there interaction between the polymorphisms and fruit and vegetable consumption in the occurrence of adenomas?

EPIDEMIOLOGICAL PERSPECTIVE: SUBJECT MATTER

Genotype – adenomas (Q1). As expected, the polymorphisms per se were not associated with a difference in adenoma risk, the NQO1 polymorphism being an exception because a substantial part of the study population consists of smokers and exposure to cigarette smoke is one important carcinogenic exposure to render the effect of the polymorphism visible.

Genotype – fruits & vegetable interaction and adenomas (Q2). Higher intake of total fruits and vegetables or cruciferous vegetables was associated with an increased adenoma risk, as has been reported in some other populations before[61-65], especially in those with genotypes conferring lower enzyme capacity. There are (at least) two conceivable biological explanations for this. One is, that fruits and vegetables may contain substances that increase the need for detoxification, which cannot be achieved sufficiently due to genetic predisposition. Another is that fruits and vegetables may contain substances that lower detoxification enzyme levels, which then reach critically low levels in those who are already genetically predisposed to lower levels.

Starting with the latter explanation, vegetable constituents may lower the expression of GSTs and NQO1 (chapter 2 and 4). The tissue-specific distribution of

the enzyme and the sensitivity of the enzyme to the other carcinogens that the population is exposed to will then further determine the vulnerability of the tissue to develop cancer. Thus, e.g., GSTP1, which might be downregulated, will be important in the colorectal area because it is the most abundant GST there. An adenoma risk difference was seen with *GSTP1* and *GSTA1* polymorphisms, and not with *GSTM1* and *GSTT1* polymorphisms. It is possible that to a certain extent a lack of capacity of a biotransformation enzyme can be compensated for by another enzyme with respect to exposures that they are both sensitive to, either from its own family (eg other GSTs[66]) or from another family (e.g. other quinone reductases[67]). If true, genotype-phenotype relations are hard to establish.

The other explanation mentioned above is that fruits and vegetables may contain substances that increase the need for detoxification. Fruit and vegetable constituents can induce phase I enzymes[49], which may then activate carcinogenic metabolites[68]. But even the phase II GST and NQO1 systems are known to be active systems towards some specific chemical classes, i.e. they may sometimes result in the formation of active carcinogenic metabolites[69]. Thus, even when substances are supplied that monofunctionally induce phase II enzymes, such as sulphoraphane from broccoli, there may still be a downside to inducing phase II enzymes and some authors warn against this[70-72] or direct possible toxicity of naturally occurring plant components[73-76].

It is conceivable that a number of commonly consumed vegetable subtypes in the Netherlands express some of the above mentioned characteristics, as explained in chapters 3 and 5, which will become visible when detoxification capacity is already genetically lower. Along the same lines, the combination of fruit and vegetable consumption and smoking, i.e. a higher carcinogen load, was particularly unbeneficial.

EPIDEMIOLOGICAL PERSPECTIVE: METHODOLOGY

Colorectal adenomas. Adenomas are not carcinomas and most (85%) never will be[77, 78]. The unspecific nature of their relation to carcinomas may be seen as a disadvantage, because it may dilute the association with risk or preventive factors for cancer. On the other hand, they provide insight into the temporal relationship between exposure to fruits and vegetables and colorectal cancer[60]. Fruit and vegetable components may block or suppress carcinogenesis in many ways; by stratifying for GST and NQO1 genotype, we focussed on mechanisms related to their detoxification potential. Detoxification potential is probably most important in the first stages of carcinogenesis, where the multiple hits by carcinogens that increasingly disorganise the replication of the cell can still be prevented. If this

mechanism plays a role of importance, it is reasonable to assume it be visible on intermediate markers such as adenomas. Some subtypes of adenomas (e.g. villous and large adenomas) are thought to confer a higher chance of carcinomas[79, 80] and thus the dilution effect will be less. Unfortunately, because our research questions already required (multiple) stratification, there was no power for elaborate adenoma type subgroup analysis.

Assessment of fruit and vegetable consumption by FFQ. In the case-control study, fruit and vegetable consumption was measured by food frequency questionnaire (FFQ), with the purpose to estimate habitual intake in the period before the endoscopy of inclusion (controls) or before the occurrence of the first adenoma (cases). Fruit and vegetable consumption was measured as a summary variable to reflect total exposure to anticancer compounds, and also cruciferous vegetable intake was assessed.

Recall bias. Cases consumed a (slightly) higher amount of vegetables than controls. A methodological explanation for this is information bias due to differential recall. Adenomas are reported to be mainly asymptomatic and thus the chance of a change in dietary habits due to symptoms is naturally small. It is possible that the recall of some cases was influenced because they knew the diagnosis before they filled out the questionnaire. This is probably limited, because gastroenterologists do not give dietary advice with respect to fruits and vegetables. Also, leaving recurrent cases out of statistical analyses in chapter 3 did not change conclusions, although this approach leaves in the new cases who filled out the FFQ after diagnosis and does not exclude some degree of recall bias. Controls may have been influenced by the presence of gastro-intestinal symptoms, causing them to start avoiding certain vegetables. Change of diet was, however, checked by our questionnaire, and only very few controls (or cases) responded affirmatively.

Exposure misclassification. It has been argued that epidemiological studies do not find consistent associations with fruits and vegetables because of the considerable variation in bioactive content between the different plant cultivars and due to the many ways and conditions of processing, storage, and preparation[81]. The consequences of this uncertainty in intake on the outcome of epidemiologic cohort studies have been simulated by Monte Carlo simulations[82]. These simulations showed that even if one assumes a strong protective effect of glucosinolates in cruciferous vegetables (or any component in fruit and vegetables) against cancer, this effect would not be statistically significantly assessed in an epidemiologic study with food intake as the main input. Thus, this would attenuate a negative association towards null. However, it will not likely result in a spurious positive association and does not explain increased risk. So far, some food

frequency questionnaires have differentiated between raw and cooked vegetables, but production, processing, storage and preparation have not been incorporated yet. It is expected that future studies will attempt to take this into account. Also, precision can be gained through the field of biomarkers, as a more integrated measure of the actually consumed, absorbed and metabolised compound. Analysis of for example glucosinolates in blood plasma is becoming a more realistic support of dietary intake data[83].

Confounding. High cruciferous vegetable consumption may be indicative of some other component that is related to the consumption of crucifers but not the causal component in the adenoma risk increase (confounding). For example, it could be an indicator of the traditional dietary pattern consuming meat, potatoes and cooked vegetables (in which case glucosinolates would probably not be the culprit as their bioavailability is severely reduced by boiling[84]). It was evaluated statistically if total, red or processed meat and low education as a proxy for social status were confounders, which they were not by our standard of a 10% change in OR, making confounding less likely.

Assessment of gene-environment interaction. A large amount of gene-environment studies have been published in the cancer field over the past years[85]. Researchers have been guided through the design and analysis of gene-environment studies in recent years[86-90] and publication criteria have been suggested[91-93]. One, if not the most, important parameter in gene-environment studies, is the estimate of interaction. Interaction is usually defined as statistical interaction and assessed by parametric methods (linear and logistic regression) as the deviation from additivity or (most commonly) multiplicativity[88]. This is meant to be a reflection of biological interaction. However, biomolecular interactions do not always result in statistical interactions, and statistical interactions do not necessarily imply underlying biomolecular interactions and thus it is not always clear what a reported interaction, or lack of it, means[94]. Some authors propose a systems biology approach to obtain the relevant information to be able to mathematically model biomolecules with respect to interindividual phenotypic differences[94]. However, sporadic cancers may strongly be subject to chance and thus a deterministic approach attempting to accurately predict risk based on complete knowledge of genetic susceptibility, even if possible, may not be appropriate.

A thorny issue in genetic association studies is the large number of polymorphisms under study by the scientific community in combination with the general reliance on significance levels of 5%[95, 96]. The probability that at least one study finds a 'significant' result is high[96] and thus it is important that scientific

journals do not focus solely on 'new' findings. Studies investigating only few polymorphisms are being replaced by those evaluating large numbers of polymorphisms, and now chips of 384, 768, up to even 500.000 SNPs have become a possibility[97]. As with all '-omics' data, the problem is analysing statistically these large numbers of polymorphisms[98]. The conventional parametric methods experience difficulty in dealing with the high risk of false positives when studying polymorphisms (multiple testing), with the large amount of genetic variation (high-dimensionality data), with clusters of genes underlying disease (genetic heterogeneity). A number of non-parametric methods have been developed, examples are MDR, GMDR, set association, random forests, neural networks. An introduction to these methods has been given by Heidema et al.[99]. At present, the best strategy may be to apply a combination of parametric and non-parametric methods and to seek replication of findings in different populations.

Population. ***Group comparability.*** In epidemiology, important concepts in aiming to discover the determinants of human health and disease are population thinking and group comparisons. Case-control studies, retrospectively comparing exposures among those with and without the disease of interest, are prone to recall and selection bias. Recall bias was addressed under 'assessment of fruits and vegetables' and is not considered to be a great problem. Selection bias, however, may have occurred in the case-control study (as discussed in chapters 3 and 5). Endoscopy controls more often experience bowel problems than cases, and this may be related to factors not truly related to absence of adenomas. Another possible source of selection bias is low response in some endoscopy-clinics. This option cannot really be excluded. An important question here is whether methodology can explain differences in ORs between genotypes. With respect to selection bias, it is thought that valid estimates of gene-environment interactions are still possible when the genotype does not influence selection conditional on exposure and disease status[100, 101]. In our case, it seems reasonable to assume that genotype did not influence participation in the study. Thus, interaction estimates may be more valid than main effects.

Also, the proportion of men and women differed between cases and controls, which may be relevant as there are some indications that men and women differ in colorectal cancer etiology even though the incidence is approximately equal. Another possible problem is the age difference between cases and controls, which may have resulted in misclassification of outcome, because cases are on average 8 years older than controls and this is a sufficient amount of time to develop an adenoma.

Generalisability. In our case-control study, controls may not be representative of 'all those without adenomas' because of referral to outpatient clinics based on abdominal problems and possible difference with population controls on genetic and environmental factors that were not measured. However, it is likely that this is not related to our research questions. Advantage of our endoscopy controls is that the absence of polyps was checked. A random population sample undergoing endoscopy would be preferable, but this would be very invasive, financially unfeasible and possibly a problem ethically. Asymptomatic controls indicated for colorectal cancer screening would also be attractive controls if attendance rate is high. Preparations for a national colorectal cancer screening programme in the Netherlands are in an advanced stage[102]. Screening will have the secondary benefit of allowing the conduct of inherently methodologically better studies.

EPIDEMIOLOGICAL PERSPECTIVE: CONCLUSION

High fruit and vegetable consumption in combinations with low-capacity *GSTP1*/*GSTA1* and *NQO1* genetic variants may slightly increase colorectal adenoma risk. A *GST* or *NQO1* genotype effect, if true, may exist in combination with certain fruit and vegetable subtypes only and one can speculate that this may be counteracted by a varied diet. Methodologically, it cannot be ruled out that fruits and vegetables actually confer some unfavourable effects in certain subgroups, but it also cannot be ruled out that some unknown confounding factor or selection bias plays a role. However, the study results do not support a protective effect from fruits and vegetables in our population.

SOCIETAL PERSPECTIVE: A REFLECTION

Definition. The societal perspective is considered to include a comparison of the general population and high-risk genotype subgroups as the subject of specific nutritional interventions with respect to public health and ethics.

Concept. In the last decades, public health has largely been practiced from a population approach, as opposed to a high-risk approach. The rationale for this is that modest behavioural changes on a population level are likely to lead to a greater public health impact than larger behavioural changes restricted to a small high-risk group[103, 104]. The Human Genome Project (HGP), and the realisation that behavioural changes on a population level are hard to accomplish, has brought debate to this approach. The genomics- and related technologies have been rapidly finding their way into nutrition research[105] and public health issues[106, 107]. One of the consequences for public health could be the adjustment of population dietary

recommendations based on genetic make-up[108-110] or even population screening for those at high-risk[111]. Efforts are being made to establish and evaluate a systematic, evidence-based process for assessing genetic tests and other applications of genomic technology in transition from research to clinical and public health practice[112]. So far, these approaches have not been put into practise by public health professionals for combinations of common polymorphisms, chronic disease and dietary prevention measures. On an individual level, however, knowledge obtained in gene-diet studies has already been applied for purposes of individual genotype-based dietary counselling, so-called tailor-made diets or personalised nutrition. Several market parties offer genetic testing (including the *GSTP1*, *M1* and *T1* polymorphisms) and subsequent dietary advice through the internet. This has raised concern for their consumers[113], as its scientific basis is widely regarded as not sufficient yet[114]. Not only are there pragmatic and theoretical issues on the road to tailoring nutritional advice to high-risk groups or individuals based on genetic make-up for preventive purposes. There are also a wide range of ethical, legal and social implications.

Reflection. Below, some issues will be reflected upon with respect to the question whether is it sensible, in the prevention of colorectal cancer, to have fruit and vegetable advice based on high-risk genotype, compared to a no-genotype population approach. This will also be done with respect to the issue of ethical justifiability of high-risk genotypes.

PUBLIC HEALTH IMPLICATIONS

Theoretical issues. With respect to our studies and our detoxification systems, GSTs and NQO1, information that contributes to tailor-made dietary counseling on a genotype basis for colorectal cancer prevention was not delivered. In a larger picture, there is not yet sufficient scientific knowledge to know the holistic effect of fruits and vegetables, neither in the form of dietary patterns nor of separate fruit and vegetable constituents, on human biology. *In vitro* assays may show a 2-3 fold induction of a detoxification enzyme by a certain plant constituent, but the effect for whole individuals or populations is not known[115] and should be carefully considered[116]. Fruits and vegetables contain an abundance of bioactive compounds (estimated number: >10.000) with possibly opposing effects. An enormous amount of enzymes play a role in carcinogenesis[117], as well as an enormous amount of genetic variation in genes encoding these enzymes, and these all influence each other. Under the heading of 'systems biology', great research efforts are being made to elucidate the complex relationships between genotypes and phenotypes in an individual through a hierarchy of biochemical, metabolic and physiological

systems that are driven by biomolecular interactions (which has been termed 'epistasis')[94, 118-120]. This will undoubtedly lead to new and better insights. However, it is not clear yet whether the holistic approach should be regarded as a solution or whether it suffers from an intrinsic problem; it is a very realistic scenario that in the end-conclusion will be that too many variables play a role to uncover the phytochemical-cancer associations in real human life for nutritional interventions directed at genotypes.

Pragmatic issues. Some authors are sceptical about the use of genomics in prevention, reasoning that if environmental influences can be modified, then genetic susceptibility looses relevance[121-123]. A populations approach to prevention of highly modifiable disease will achieve a greater public health benefit than intervention targeted to high-risk groups on the basis of genotypes. However, the population strategy comes with the disadvantage of a small benefit for the individual (the so-called 'Prevention Paradox') and poor motivation[104] . It has been proposed that knowledge of genetic make-up could be a stronger motivation for people to change to good nutrition behaviour. Incorporation of genetic susceptibility feedback has been evaluated in relation to smoking cessation[124-126]. Unfortunately, it did not have the desired behavioural change. This may also be true for nutrition behaviour[127]. One can speculate that different high-risk approaches are likely to produce different results, because they require a more rational or more emotional (e.g. in the case of a positive family history) weighing of costs and benefits of changing behaviour. More research into behavioural changes is needed. At the same time, we must not lose the ideals of changing society's norms of behaviour (instead of only high-risk individuals). More funds should be allocated to changing society's infrastructure with respect to physical activity and the availability of (un)healthy food.

Defining high-risk groups or high-risk individuals also has a range of social, legal and ethical implications. Below, some of the ethical implications are addressed.

ETHICAL IMPLICATIONS OF INVESTIGATING INTERACTIONS BETWEEN DIETARY AND GENETIC FACTORS. (For a background related to the project described in this thesis, see addendum V). Early writings on the human genome project warned to take social, legal and ethical issues seriously and protested the low budget for exploring these[128-130]. According to one source, in 1989 we were "utterly unprepared to deal with issues of mandatory screening, confidentiality, privacy and discrimination. The genome project has been overhyped and oversold". Over ten years later these issues are still subject of concern[131-133].

Many scenarios can be imagined with respect to preventive diets tailored to genotype information, with varying ethical (and legal and social) consequences, depending for example on how drastic the suggested dietary changes are, how great the estimated benefit is and whether the genetic test is on an individual, self-initiated basis or part of a screening program. Let's imagine that the scientific community has identified a set of genotypes so that:

-those with set A benefit from regular consumption of vegetable subtype X with respect to colorectal cancer prevention (35% risk reduction) and

-those with set B do not benefit.

Now let's reflect upon some of the ethical consequences, from a societal viewpoint. One obvious consequence is that the relation between food and health or disease is further complicated by medical terminology such as 'lowered genetic susceptibility', and will contribute further to the so called *medicalisation* of nutrition[134]. With an increasing number of available tests, less and less individuals will perceive themselves as healthy individuals. Also, including personal genetic information to preventive advice adds a new dimension of risk communication. Most challenging may be to explain to group B that eating vegetables (including vegetable X) may still help them to prevent other diseases than colorectal cancer.

In our situation, the test is on the market. The choice, then, to obtain the test should be with the consumer. "Individuals are, ultimately, the best judges and guardians of their own interests" as formulated by J.S. Mill, a well-known utilitarian (1859, 'On Liberty'). This is essentially the concept of personal *autonomy***:** the right of an individual to act freely. The importance of autonomy has been shown[135-137] and as flourishing individuals better contribute to the general good, autonomy fits well within the utilitarian way of thinking. For autonomous consumership to "work", well-communicated, objective information is needed. The information flow is ever increasing, making it impossible for consumers to keep up. It has to be believed that individuals can actually absorb this information and base choices on it. This modern consumer-citizen may be an illusion, people may not use their autonomy optimally; But it would in general seem better that people made their decisions on the basis of knowledge rather than from ignorance.

However, knowledge is not the only ingredient that individuals base choices on. There may be good reasons for not adhering to the advice, on different grounds. Consumption of food is a *social event*, as consumption and type of food are very much embedded in our family- and local environment. It gives shape to the *identity* of many individuals with certain convictions[138]. Tailoring food to the individual genetic make-up may divide families and social gatherings and distort the fulfilment of eating according to one's religious or other beliefs. On a different

level, some members of group A may have a genetically determined dislike for vegetable X[139, 140]. Once the test results are there, deliberately not complying to a diet may lead to feelings of *guilt* and unrest[141], and thus to a diminishing of well-being. This leads to issues whether non-complying individuals may be held accountable when they do get ill (e.g. by health insurance companies). Also, genetic knowledge may lead to some problems in families, because autonomy of one individual to know personal genetics may clash with a family member's autonomy not to know this.

On the other hand, in western societies health is often considered a right, which is society's responsibility. Yet it can be argued that consumers have a moral responsibility to make some sacrifices in order to lower the disease burden and maintain a social health care system based on solidarity. Blaming and sense of individual responsibility per se are acceptable, however it does not seem fair to preclude this to individuals with certain (unchangeable) genetic make-up. Adopting general nutrition and physical activity guidelines may suffice.

Attention needs to be given to a big player in genetic testing and society at large: industry. It has made great investments in genetics/genomics, is the first to offer applications in the form of genetic testing through the internet and increasingly participates in science[142]. In society's interest, some rules and regulations for market parties are needed. If pills or whole foods (by genetic modification) are concentrated with the phytochemicals that are presumed to be responsible for the effect of vegetable X, possible adverse effects need to be monitored. These can be, for example, overdoses, adverse combinations with other foods of medication, or not-foreseen impact on unknown pathways that become apparent after large-scale introduction.

Though ultimately perhaps beneficial to health, it is not so self-evident that tailor-made dietary advice is overall beneficial to our well-being.

SOCIETAL PERSPECTIVE: CONCLUSION

At his point, it is not self-evident that polymorphisms will have a legitimate place in nutritional prevention strategies. Their functionality on the societal level has not been shown. In addition, the efficiency and efficacy of high-risk groups based on genotype is still under debate. Also, identification of high-risk groups or individuals, especially based on genetic information, comes with valid ethical issues, adding an extra dimension of reflection to the issue of individual nutritional preventive strategies.

FINAL REFLECTION

The relation between fruit and vegetable consumption and protection against carcinogens in cancer prevention is complex. Many variables play a role: there is a large array of polymorphisms, epigenetic processes, biotransformation enzymes, bioactive plant components, other dietary variables and environmental influences (among which different (pro)carcinogens), possible doses, tissues, microbiotal influences and, expectedly, synergy and antagonism. The long-term effects for the whole organism of single substances or entire dietary patterns are not yet understood. Even if the interactive effects of the above variables could be mapped and understood, there would still remain an element of chance which individual would develop cancer. In addition, many other mechanisms besides biotransformation are likely to play a role in the prevention of cancer by fruits and vegetables, such as by scavenging reactive oxygen species and by influencing DNA repair and apoptosis, increasing the number of possible associations even further. Also, there are many life processes and thus disease etiologies, e.g. in hypertension and diabetes, in which fruits and vegetables are thought to play a role. Given the above, there is currently no reason to exchange the population advice of high, diverse, daily fruit and vegetable consumption, for genotype-specific and phytochemical-specific advice.

FUTURE RESEARCH

Biotransformation induction by fruits and vegetables and genetic variability.

The question remains whether there is a real net beneficial effect of fruit and vegetable consumption on the detoxification potential of the colorectal area *in vivo*, which plant subtypes or compounds are most beneficial and what the role of genetic variation is in this. This will not be solved by single intervention studies uncoordinately focussing on single vegetable types and single genetic polymorphisms. A series of *systematically* designed human feeding trials can provide a more complete picture. Different types of vegetables, vegetable constituents, doses, exposure times, phase II enzymes, phase I enzymes and polymorphisms would ideally be included. The obvious difficulties are that they are expensive, and the possible combinations of the above are endless. A large-scale collaboration could be a good framework, each center performing the part on its own expertise, thus uniting knowledge and standardizing methods. Outcomes should include but surpass the level and activities of the biotransformation enzymes (which could be measured by means of an X-omics approach[115, 117] to take the many possible effects into account[97] , with a focus on the protein level, as the correlation with RNA is generally low[143]) and take functional effect-measures of

possibly increased detoxification potential of the colorectum into account (e.g. urinary markers of carcinogen metabolism and markers of DNA damage).

Crucifers and glutathione S-transferases. The protective effect against colorectal cancer of high consumption of cruciferous vegetables among those with genetic variants associated with lower GST detoxification potential (as investigated in chapter 3), remains debatable. There are clues that the effect may differ between crucifer types. Thus, detailed questionnaires separating crucifer types, or crucifer subtype-specific biomarkers, are required. Ideally, large (new and existing) cohorts could be used to reduce selection and information bias and have the power needed for high-dimensional genotype analysis, taking the various GSTisoenzymes into account. Finally, other cancer sites need to be taken into account, because higher intake of a certain vegetables may affect tissues differently.

Validity and reproducibility of measuring fruit and vegetable consumption in epidemiological studies. The validity and reproducibility of fruit and vegetable assessment in epidemiological studies may not be sufficient. Questionnaires incorporating questions on growing, storing and preparation of fruits vegetables may improve precision. However, information bias due to the 'subjectivity' of the subjects filling out the questionnaires cannot be avoided (although it can be somewhat lessened by avoiding those with disease states affecting their answers, such as can be done in cohort studies). Thus, the search for valid and reproducible biomarkers for the intake of (different types of) fruits and vegetables in small-scale human trials needs continued effort. Biomarkers have the additional advantage that they do not only represent intake, but can also be a measure of absorption and metabolism (in which not to forget genetic variation). If valid and reproducible links can be established between intake and marker, then epidemiological studies will have higher power. The most precise and valid results are expected from large, prospective populations in large scale collaborations[144, 145], in which biobanks are extremely valuable[146, 147] to enlarge power or provide posterior mechanistic back-up of exploratory findings.

The use of more integrated measures of diet such as dietary patters can give a more balanced representation of pro- and anticarcinogens.

In general. The ability to sequence genomes and the array of technologies that developed from this, is giving a whole new dimension to epidemiology (and public health). Communication between the different disciplines is highly important. The epidemiologist must not discard a certain degree of skepticism towards the increasing bulk of data that is being generated, but must also be open-minded.

SELECTED WEBSITES OF INTEREST

-http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml
(human genome project)
- http://www.ornl.gov/sci/techresources/Human_Genome/elsi/elsi.shtml
(social, legal and ethical implications in hpg)
-www.ncbi.nlm.nih.gov (public database)
    -SNP
    -dbGaP *(db of genotype-phenotype studies)*
-www.hapmap.org
(HapMap project)
-www.cdc.gov/genomics/HUGEnet.default.htm
(Human Genome Epidemiology network)
- http://www.cdc.gov/genomics/gtesting/EGAPP/about.htm
(Evaluation of Genomic Applications in Practice and Prevention)
-http://www.hupo.org/
(human proteome project)
- http://www.epigenome.org/
(human epigenome project)

ADDENDA

1) Study findings, elaborate version
2) *GSTA1*, *GSTP1* and *NQO1* DNA sequences
3) Associations between *NFE2L2* polymorphisms and GST phenotype
4) Botanical definition of vegetables
5) Background to project and ethics

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Addenda to the General Discussion

Table 1.1: Main effects of fruit & vegetable consumption and polymorphisms on colorectal enzymes and adenomas

Endpoint	Determinant[a]	Association with endpoint	Chapter
General			
Colorectal adenoma risk	Total fruit and vegetable consumption	↑ tertile 2 and 3 to 1	5, table 6
Glutathione-S transferases (GSTs)			
Rectal total GST activity	*GSTP1* c.313A>G	↓ and ↓↓ for AG and GG genotypes compared to AA	2, table 4
	GSTP1 g.272C>G	↑ for GG genotype compared to CC	
	GSTA1 g.-69C>T	↑ for CT and TT genotypes compared to CC	
	Fruit or vegetable consumption	↔ for yes compared to no consumption (except fruit and GSTM1 null)	2, table 5 + text
(+GSTP1 313 SNP in model)	Alliaceae consumption	↑ for yes compared to no consumption	2, text
Rectal GSTM1 level	Citrus fruit + juice consumption	↑ for yes compared to no consumption	2, table 3
	Cruciferae consumption	Not sign: ↑ for yes to no consumption	
Rectal GSTM2 level	Total vegetable consumption	↓ for yes compared to no consumption	2, table 3
	Apiaceae consumption	↓ for yes compared to no consumption	
Rectal GSTT1 level	Alliaceae consumption	↑ for yes compared to no consumption	2, table 3
	Cruciferae consumption	Not sign: ↑ for yes to no consumption	2, table 3
Rectal GSTA level	*GSTA1* g.-69C>T	↓ and ↓↓ for CT and TT genotypes compared to CC	2, figure 1
(+GSTA1 SNP in model)	Solanaceae consumption	↑ for yes compared to no consumption	2, text
(+GSTA1 SNP in model)	Cucurbitaceae consumption	↑ for yes compared to no consumption	2, text
Rectal GSTP1 level	*GSTP1* c.313A>G	↑ and ↑↑ for AG and GG genotypes compared to AA	2, text
	GSTP1 g.217G>A	↑ for GA genotype compared to GG genotype	2, text
	Alliaceae consumption	↑ for yes compared to no consumption	2, table 3
	Cucurbitaceae consumption	↑ for yes compared to no consumption	2, table 3

NAD(P)H quinone:oxidoreductase (NQO1)

Rectal *NQO1:β-actin* mRNA ratio	*NQO1* c.609C>T	↑ for CT genotype compared to CC genotype	4, figure 1 & table 3
	NFE2L2 g.-684 G>A	↑ for GA genotype compared to GG genotype	4, table 3
	NQO1 c.609C>T and *NFE2L2* g.-684 G>A	↑ for *NFE2L2* GA compared to GG genotype among *NQO1* 609CC	4, table 4
	Total vegetable consumption	↓ for yes compared to no consumption	4, table 2
	Compositae consumption	↓ for yes compared to no consumption	4, table 2
Rectal NQO1 enzymatic activity	*NQO1* c.609C>T SNP	↓ for CT genotype compared to CC genotype	4, figure 1 & table 3
	Fruit or vegetable consumption	↔/ ↓ for yes compared to no consumption	4, table 2 & 5
	Apiaceae consumption	↓ for yes compared to no consumption	4, table 2
Colorectal adenoma risk	*NQO1* c.609C>T	↑ for NQO1 CT genotype compared to CC genotype	5, table 3
	NQO1 c.609C>T and *NFE2L2* -686A>G	↑ for *NQO1* CT/*NFE2L2* AG and *NQO1* TT/ *NFE2L2* GG[b]	5, table 4
	NQO1 g.-718G>A and *NFE2L2* g.-650C>A	↑ for *NQO1* GA and *NFE2L2* CA combined[b]	5, table 4

[a]no data on GSTP1 g.217G>A, GSTP1 g.227G>A and GSTP1 g.272C>G SNPs in adenoma study

[b]compared to both common variants

Table 1.2: Interactive effects fruit & vegetable consumption and polymorphisms on colorectal enzymes and adenomas

Endpoint	Determinant[a]	Association with endpoint[b]	Chapter
Glutathione-S transferases (GSTs)			
Total GST activity	Total fruit consumption and *GSTM1*	↑ for yes consumption, among *GSTM1*-	2, text
GSTP1 level	Alliaceae and *GSTP1* g.272C>G	↑ for yes consumption, among *GSTP1* 272CC genotype	2, text
	Cucurbitaceae and *GSTP1* g.272C>G	↑ for yes consumption, among *GSTP1* 272CC genotype	2, text
Colorectal adenoma risk	Cruciferae consumption and *GSTP1* c.313A>G	↑ for high consumption among *GSTP1* 313 GG	3, table 2
	Above and *GSTA1* g.-69C>T	↑ for high consumption among *GSTP1* 313GG or *GSTA1* -69TT	3, table 2
	Cruciferae consumption, *GSTP1* c.313A>G and age	↑ for high consumption and age>55 years among *GSTP1* 313 GG	3, table 3
	Cruciferae consumption, *GSTP1* c.313A>G and sex	↑ for high consumption and male among *GSTP1* 313 GG	3, table 3
	Cruciferae consumption, *GSTP1* c.313A>G and processed meat	↑ for for high consumption and high processed meat among *GSTP1* 313GG	3, table 4
	Cruciferae consumption, *GSTP1* c.313A>G and smoking	↑ for high consumption and never smokers among *GSTP1* 313GG	3, table 4
	Cruciferae consumption, *GSTA1* g.-69C>T and age	↑ for high consumption and <55 years among *GSTA1* -69TT	3, table 3
	Cruciferae consumption, *GSTA1* g.-69C>T and red meat	↑ for high consumption among *GSTA1* -69TT and high red meat	3, table 4
NAD(P)H quinone:oxidoreductase (NQO1)			
NQO1:β-actin mRNA ratio	Total fruit consumption and *NQO1* c.609C>T	↓ for yes consumption, among *NQO1* 609CC genotype	4, text
	Total vegetable consumption and *NQO1* g.-718 G>A	↓ for high consumption among *NQO1*-718GA genotype	4, text
	Compositae and *NFE2L2* g.-650C>A	↓ for yes consumption, among *NFE2L2* -650CC genotype	4, text
NQO1 enzymatic activity	Total fruit and vegetable consumption and *NFE2L2* g.-686A>G	↓ for high consumption among *NFE2L2* -686AA genotype	4, table 5
	Compositae and *NFE2L2* g.-686A>G	↓ for yes consumption, among *NFE2L2* -686 AA genotype	4, text
	Total vegetable consumption and *NQO1* g.-718G>A	↓ for high consumption among *NQO1* -718GA genotype	4, text
	Smoking and *NQO1* c.609C>T	↑ for *NQO1* 609CC and current smoking	5, text
Colorectal adenoma risk	Smoking and *NQO1* c.609C>T	↑ for *NQO1* 609CT and smoking	5, table 5
	Total fruit and vegetable consumption and *NQO1* g.-718G>A	↑ for high consumption among -718 GG ↔ for high consumption among -718 GA	5, table 6
	Smoking, total fruit and vegetable consumption and *NQO1* c.609C>T	↑ for smoking and high fruit and vegetable consumption and *NQO1* 609CT	5, text
	Smoking, alcohol consumption and *NQO1* c.609C>T	↑ for smoking and highest tertile of alcohol and *NQO1* 609CT ↑ for non-smoking and highest tertile of alcohol and *NQO1* 609CT	5, text

[a]no data on GSTP1 g.217G>A, GSTP1 g.227G>A and GSTP1 g.272C>G SNPs in adenoma study

[b]compared to low/no consumption and/or most common variant

Below, in the genomic sequences for the genes under study (i.e. GSTA1, GSTP1 and NQO1), are indicated:

-the sequence primers used for sequencing the regulatory areas

-the SNPs that were studied in this thesis

Gene: **GSTA1**
Genbank accession no. **L13269**
[Human glutathione S-transferase PJ-1A pseudogene, exons 6 and 7; glutathione S-transferase A1 (GSTA1) gene, exon 1, total sequence 1-4786]

ORIGIN

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2761 tattgaagag caaacagacc caccctctgc tctcaggcca acttgtcatc ccttacagtg
2821 catgtgctcc tggctcatcc tcactctcct caaggctctg tggggacccg ggcatgagct
                    ------------------→ GSTA1-prom-fw
2881 tccctcagtt cgttcagtgc cactcaggcc tggctgcgaa gatacagatg gtatatgcga
2941 attacatata agtaactata tgcaaaatta tttttccctg gtgtgtcatt gtgccagcgt
3001 gtttggatta gaagtgattt ccaggaatcc ttccctgcgt cttctagctt ctgggggctc
3061 ttggctttct ggactttcat ggatcatgac cacatcactc taatttctgc ctctgtgttc
3121 cagcacctca ccccctgtgt gttgtgtctt ctcttctctc tgctgtgagg acaattgtca
3181 ttggatttgg ggcctatcca gatataccaa gatgatctca tctcaaattc ttttacttca
3241 ttacatctgc aaagagcctt tttccaaata agatcaagtt cacaggatct agggatttct
3301 atatggacct atctttcttg ggggccaaca taacccccta catggtatag gtgaaataag
3361 attatttgca attgatgaat ccaggtgatg aatactttca gatttgttat attatgggca
3421 atttgagtga cgcaaagagg atagcatatg caaatagggt ctctggattt gtcagataaa
3481 atacaggata cccacctaaa tgtgaatttc agataaacaa taaataatat ttcagtataa
3541 tctggtttca taccgtgttt ggaacatagc tataactaaa aaattgttga ttgtttgcct
3601 gaaattcaca tttacatgga tgtcctatat tttatttggc aacccagtaa gaaaacggtg
3661 gcatcagctt gcccttcaca gacatcctct cccagctatg ctcacagtag agatttttca
3721 gttgccctag tctttgcacc caactcatga aaaaaagcat ctttaaaaag ccagtttctg
3781 ctgacttgca aaaagagcaa aatctcggtg aaatgtattg tgtaaacttt gattgccaac
3841 cttgaaaagg aacacattaa ccagtttctt ctgataagca gatcacttgc ctcatgtctt
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3961 ccttctttc agtgggaggg aactattgag aggaacaaag agcttataaa tacattagga
      t-69              a-52
4021 cctggaattc agttgtcgag ccaggacggt gacagcgttt aacaaagtaa gtactgatct
                              ←-------------- GSTA1-prom-rv
4081 tataaatttc tctacattgc cttacaccct cctgtctagc ctcctagaaa aatatgctaa
```

Gene: **GSTP1**

Genbank accession no. **AY324387**

[Homo sapiens glutathione S-transferase pi (GSTP1) gene, complete cds, total sequence 1-5635]

Transcription initiation site: 1887

Translation start (ATG): 1916 (A) and 2207+2208 (T+G)

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ORIGIN
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  1081 taaaagttgg acggccaggc gtggtggctc acgcctgtaa tcccagcact ttgggaagcc
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                                                      g 313(exon 5)
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  3661 cacttttagc tgaggaaaat atgcagacac agagcacatt tggggacctg ggaccagttc
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```

Cauchi et al.[1]: SNPs at position: -282, -287, -289, -354 relative to transcription initiation site

1. Cauchi S, Han W, Kumar SV, Spivack SD. Haplotype-environment interactions that regulate the human glutathione S-transferase P1 promoter. Cancer Res 2006;66:6439-48.

Gene: **NQO1**
Genbank accession no. **M81596**
[Human NAD(P)H:quinone oxireductase gene, exon 1], total sequence
Transcription initiation site: 1824
Translation start (ATG): 1935

ORIGIN

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                        fw primer ---------------→
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  2641 aatttt
```

(coding region continues at genbank accession number M81597; contains *NQO1* c.609C>T SNP)

Table 1: Rectal GST isoenzyme levels (ng/mg protein) by *NFE2L2* polymorphisms

		n[a]	GSTM1		GSTM2[b]		n[a]	GSTT1		n[b]	GSTA		GSTP1	
Consumption[c]		45	reference mean[*] +/- difference (95% CI)	p-value	reference mean[*] +/- difference (95% CI)	p-value	74	reference mean[*] +/- difference (95% CI)	p-value	92	reference mean[*] +/- difference (95% CI)	p-value	reference mean[*] +/- difference (95% CI)	p-value
NFE2L2	CC	40	2132±193		1335±73.1		65	4737±319		80	463±43.3		6555±279	
g.-650C>A	CA	5	+124 (-1023, 1270)	0.83	-48.2 (-464, 367)	0.82	8	-645 (-2554, 1263)	0.50	11	+52.9 (-193, 299)	0.67	-572 (-2159, 1015)	0.48
	AA	-	-		-185 (-1494, 1124)	0.78	1	-463 (-5629, 4703)		1	+775 (-0.01, 1551)	0.050	-344 (-5339, 4651)	0.89
NFE2L2	GG	33	1931±200		1271±77.5		56	4796±339		69	508±47.0		6532±301	
g.-684G>A	GA	12	+839 (20.3, 1657)	0.045	+252 (-59.9, 563)	0.11	17	+447 (-18.9, 999)	0.054	22	-116 (-305, 73.1)	0.23	-162 (-1374, 1049)	0.79
	AA	-	-		-360 (-1649, 930)	0.58	1	-2945 (-8072, 2183)	0.26	1	-483 (-1266, 299)	0.22	-873 (-5887, 4140)	0.73
NFE2L2	AA	19	2244±290		1363±111		27	4243±505		35	414±66.3		6238±428	
g.-686A>G	AG	21	-218 (-1014, 578)	0.58	-104 (394, 185)	0.48	39	+683 (-603, 1968)	0.29	47	+150 (-23.7, 324)	0.090	+383 (-732, 1497)	0.50
	GG	5	+86.3 (-1182, 13.6)	0.63	+171 (-291, 633)	0.46	8	+452 (-1628, 2531)	0.67	10	+36.3 (-242, 314)	0.80	+432 (-1348, 2212)	0.63

[*]adjusted for age, sex, sample storage time, smoking

[a]n: only those who possess the gene

[b]n is the same for GSTP1, GSTA1 and GSTM2, i.e. total population

[c]on (one of) the two days before specimen collection

RESULTS

The *NFE2L2* -684GA genotype was associated with a higher GSTM1 and GSTT1 level (Table 1). GSTA level appeared to be slightly higher with the *NFE2L2* -686AG genotype.

The *NFE2L2* polymorphisms did not significantly affect total rectal GST activity (Table 2). Total lymphocyte GST activity was significantly higher among those with the *NFE2L2* -684GA genotype (Table 2).

Consumption of Solanaceae possibly influenced GSTA level (data not shown). This was most pronounced for the *NFE2L2* -686AA genotype: consumers had a 273 ng/mg protein higher GSTA level (p=0.016) compared with non-consumers, whereas for both the AG and GG genotype there was no difference (+101 ng/mg protein, p=0.51 and +1.06 ng/mg protein, p=0.99, respectively, data not shown).

The *NFE2L2* -684GA genotype appeared to be associated with a higher raise in GSTP1 level with consumption of Alliaceae: +3151 ng/mg protein, p=0.028 (data not shown).

For citrus fruit and juice, both combined and apart, there appeared to be a difference among those with the *NFE2L2* -684GG genotype and those with the -684GA genotype; for citrus fruit and juice combined, the difference between consumers and non-consumers was +956 ng/mg protein, p=0.042 among the -684GG genotype, compared to -370 ng/mg protein, p=0.86 among those with the -684GA genotype. For Cruciferae there was a similar trend, with numbers of +878, p=0.063 and -2037, p=0.17, respectively.

Table 2: Rectal and white blood cell total GST activity (nmol/min/mg protein), by *NFE2L2* genotype

		Rectal total GST activity			White blood cell total GST activity					
					Leukocytes			Lymphocytes		
Genotype		n	reference mean* +/- difference (95% CI)	p-value	n	reference mean* +/- difference (95% CI)	p-value	n	reference mean* +/- difference (95% CI)	p-value
		92			75			93[a]		
NFE2L2	CC	80	214±6.10		65	134±4.73		81	129±5.76	
g.-650C>A	CA	11	+20.0 (-14.7, 54.7)	0.26	9	+5.54 (-21.7, 32.8)	0.69	11	+5.85 (-27.3, 38.0)	0.73
	AA	1	+40.1 (-69.2, 149.3)	0.47	1	-21.0 (-98.0, 56.0)	0.59	1	-24.3 (-129, 80.1)	0.65
NFE2L2	GG	69	219±6.57		54	135±5.22		70	123±6.07	
g.-684G>A	GA	22	-6.36 (-32.8, 20.0)	0.63	20	-1.08 (-21.0, 18.9)	0.92	22	+25.5 (1.28, 4936	0.039
	AA	1	-72.8 (-182, 36.5)	0.19	1	-23.6 (-101, 53.3)	0.54	1	-39.9 (-141, 63.0)	0.45
NFE2L2	AA	35	218±9.44		29	132±7.10		36	129±8.59	
g.-686A>G	AG	47	-1.12 (-25.7, 23.4)	0.93	4	+4.42 (-14.1, 23.0)	0.64	47	+5.72 (-16.9, 28.3)	0.16
	GG	10	-12.9 (-52.2, 26.3)	0.51	5	+3.31 (-33.7, 40.4)	0.86	10	-24.1 (-60.6, 12.4)	0.19

*adjusted for age, sex, sample storage time

[a]1 outlier deleted

Table 1: Botanically defined vegetables, English version

Botanical group*	English common*	Examples English
Liliaceae (Alliaceae)	Lily	Leek, Onion
Umbelliferae (Apiaceae)	Carrot	Celery, Carrots, Fennel
Compositae	Daisy	Andive, Oyster plant, Lettuce, Chicory, Artichoke
Cruciferae (Brassicaceae)	Mustard	Cauliflower, Kale, Broccoli, Red cabbage, Brussels sprouts, Paksoi, hodgepodge (correction factor for potato content), Radish, Garden cress
Cucurbitaceae	Gourd	Cucumber, Gherkins, Zucchini
Solanaceae	Night shade	Egg plant, Bell pepper, Tomato

*after Smith et al.[1]

Table 2: Botanically defined vegetables, Dutch version

Botanische groep	Dutch common	Examples Dutch
Liliaceae (Alliaceae)	Lookfamilie	Prei, Ui
Umbelliferae (Apiaceae)	Schermbloemfamilie	Selderij, Wortelen, Venkel
Compositae	Komposietenfamilie	Andijvie, Schorseneren, Sla, Witlof, Artisjok
Cruciferae (Brassicaceae)	Kruisbloemfamilie	Bloemkool, Boerenkool, Broccoli, Rode kool, Spruiten, Paksoi, stamppotten* (*correctiefactor voor aardappel inhoud), Radijs, Tuinkers
Cucurbitaceae	Komkommerachtigen	Komkommer, Augurken, Courgette
Solanaceae	Nachtschadefamilie	Aubergine, Paprika, Tomaat

1. Smith SA, Campbell DR, Elmer PJ, Martini MC, Slavin JL, Potter JD. The University of Minnesota Cancer Prevention Research Unit vegetable and fruit classification scheme (United States). Cancer Causes Control 1995;6:292-302.

STUDY OBJECTIVES IN ETHICAL PERSPECTIVE, A BACKGROUND

The objective of the project described in this thesis, funded under the 'innovation' subprogram of the Health Promotion and Disease Prevention Programme 1998-2002 of the Health Research and Development Council of the Netherlands (ZonMw), was to study the significance of genetic variation in certain regions of detoxification genes with respect to colorectal adenoma risk and risk modulation by various plant food components. Its ultimate goal was described in the project proposal as the provision of "the necessary knowledge on the interaction between the genotype of an individual and plant food components in order to enable genotype-based dietary counselling aimed at colorectal cancer biochemoprevention". ZonMw aims to "allow the innovation cycle to flourish and help create a cutting-edge society", by actively promoting exchange of ideas, knowledge and experience among all relevant stakeholders (identified as health researchers, health professionals, patients/consumers and the general public), along "a single 'knowledge continuum': from basic, strategic and applied research to daily health care services" (www.zonmw.nl). Thus, implementation of generated knowledge plays an important role. In the project proposal, no difficulties were expected there: "Once the required genetic and biochemical knowledge will be available, the ensuing implementation into practice will be relatively straight-forward, and will essentially involve the creation of the necessary information infrastructure allowing effective dietary guidance, expecially for those at elevated risk to develop colorectal cancer. The chances for practical implementation of such a cancer prevention strategy are particularly good, since no complex or disturbing life style intervention is needed, but only a dietary adaptation, which is likely to be well-acceptable to the general public". The introduction of such a technique, however, has ethical implications on an individual and a societal level. Realising this, ZonMW granted in subprogram 3 'implementation aspects' of the Prevention Programme 1998-2002, a research proposal by the Bioethics Institute Utrecht (Brom, Verweij and Meijboom) that deals with the ethical issues raised by the concept of personalised diets. Researchers are part of the "innovation cycle". As such, they might reasonably be attributed a responsibility to account for the ethical and societal implications of the knowledge they generate. During the stage of generating knowledge, choices are made by researchers. A second research proposal triggered by the process of studying genetic information consisted of an ethnographic exploration of normativity in biomedical research (Nelis). To what degree do choices in the research process determine the choices for consumers once the knowledge can be implemented?

Summary

Colorectal cancer is a significant cause of morbidity and mortality in the Netherlands and worldwide. Consumption of fruits and vegetables is thought to protect against the development of colorectal tumors. One possible mechanism is the induction of detoxification enzymes such as glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase (NQO1) by fruit and vegetable components. These enzymes reduce the levels of carcinogens in the cells and tissues and thus contribute to the reduction of DNA damage and cancer risk.

The strength of the inverse association is uncertain, however. An explanation for this may be the difficulty of valid and reproducible fruit and vegetable measurement. Another explanation may be interindividual genetic variation and its interaction with diet that has not been considered enough. GSTs and NQO1 are known to be polymorphic. These polymorphisms may influence the effect the presumed effect of fruits and vegetables. This may especially be true for polymorphisms in the regulatory regions of the genes, affecting individuals' responsiveness.

In previous studies, GST levels and activities have been noted to be higher by consumption of cruciferous and allium vegetables and citrus fruits. However, results are inconsistent. Rarely is more than one fruit and vegetable subtype or more than one GST isoenzyme considered, and often blood is used as a surrogate tissue. Genetic polymorphisms are usually not taken into account. For NQO1 there are little data in humans. Also, with respect to further integrated measures in colorectal carcinogenesis and prevention, such as adenomas, the interaction between polymorphisms in GST and NQO1 and consumption of fruits and vegetables is not sufficiently known. In addition, from previous research, little is known about polymorphisms in GST or NQO1 regulatory regions.

In this thesis, we evaluated fruit and vegetable consumption and different polymorphisms in *GSTA1*, *GSTP1* and *NQO1* in relation to the expression and enzymatic activity of GSTA1, GSTP1 and NQO1 in human rectum (endpoint 1), as target tissue, and white blood cells, as possible surrogate tissue. Also, we evaluated genotype-diet interactions in relation to the occurrence of colorectal adenomas (endpoint 2). This can enlarge scientific insights in aspects of carcinogenesis and cancer prevention and may lead to the identification of specific groups suitable for nutritional intervention.

With regard to the first endpoint, enzyme phenotype (i.e. mRNA level, enzyme level, enzymatic activity level), the following research questions were formulated:

1) Are genetic polymorphisms in regulatory and coding DNA sequences of GSTP1, GSTA1 and NQO1 associated with altered GST and NQO1 enzyme phenotype?

2) Is recent fruit and vegetables consumption associated with altered GST and NQO1 enzyme phenotype?
3) Does high fruit and vegetables consumption modulate enzyme phenotype in combination with specific genotypes (is there gene-environment interaction)?
4) Is white blood cell GST and NQO1 enzyme phenotype a good surrogate measure for colorectal GST and NQO1 enzyme phenotype?

To answer these questions, an observational phenotyping study was conducted among 94 sigmoidoscopy patients without colorectal inflammation or cancer. Polymorphisms in *GSTA1*, *GSTP1*, *GSTM1*, *GSTT1*, *NQO1* and *NFE2L2* were determined, recent fruit and vegetable consumption was assessed by food record, and GST en NQO1 enzyme levels and activities were measured in rectal biopsies and white blood cells.

In **chapter 2**, the results for the GSTs are described. Rectal GST isoenzyme levels differed between those with and without recent consumption of Alliaceae, Cucurbitaceae, Apiaceae and citrus fruit. Rectal GST activity, however, was not clearly influenced by fruit and vegetable consumption. It was most significantly determined by the *GSTP1* c.313A>G polymorphism; compared to the 313AA genotypes, the 313AG and 313GG genotypes showed 36 and 67 nmol/min.mg protein ($p<0.001$) lower GST activity, respectively. The correlation between rectal and white blood cell GST activities was low ($r=0.40$, $p<0.001$), and the relevance of the various genetic and dietary factors appeared to differ between the two tissues.

Chapter 4 describes the results for NQO1. Consumption of fruits and vegetables did not yield higher mRNA level or activity, but rather appeared to have a repressive effect. Rectal NQO1 activity was higher among *NQO1* 609CC-genotypes as compared to 609CT-genotypes ($p<0.0001$; 609TT-genotypes were absent), whereas mRNA was higher among 609CT-genotypes ($p<0.001$). mRNA and activity correlated among *NQO1* 609CC-genotypes ($r=0.50$, $p=0.0001$) but not among 609CT-genotypes ($r=0.14$, $p=0.45$). The *NFE2L2* -684A-allele was associated with higher *NQO1* mRNA levels ($p=<0.05$). The other polymorphisms did not affect phenotype significantly. Rectal and white blood cell NQO1 activities did not correlate.

With regard to the second endpoint, colorectal adenomas, the following research questions were formulated:
1) Are genetic polymorphisms in regulatory and coding DNA sequences of *GSTP1*, *GSTA1* and *NQO1* associated with altered adenoma occurrence?
2) Is fruit and vegetable consumption associated with altered adenoma occurrence?

3) Is fruit and vegetable consumption associated with a different adenoma occurrence between genotypes (is there gene-environment interaction)?

In a case-control study among 1477 endoscopy patients (746 adenoma cases and 698 controls), *GSTA1*, *GSTP1*, *GSTM1*, *GSTT1*, *NQO1* and *NFE2L2* polymorphisms were determined and habitual fruit and vegetable consumption was assessed by food frequency questionnaire.

In **chapter 3**, the results for the GSTs are described. High cruciferous vegetable consumption was slightly positively associated with colorectal adenomas, odds ratio (OR) 1.15, 95% confidence interval (CI) 0.92-1.44. For *GSTP1*, a positive association with higher cruciferous vegetable intake was only apparent in individuals with the low activity *GSTP1* genotype (GG genotype, OR 1.94; 95% CI 1.02-3.69), contrary to our hypothesis. This interaction was more pronounced in men, with higher age and with higher meat intake. The *GSTA1* polymorphism may have a modifying role as well: the OR for higher intake compared to lower intake was 1.57 (0.93-2.65) for individuals homozygous for the low expression variant (TT genotype). This appeared to be stronger with younger age and higher red meat intake. Cruciferous vegetable consumption and the combined *GSTA1* and *GSTP1* genotypes showed a statistically significant interaction (p for interaction=0.034). The *GSTM1* and *GSTT1* genotypes did not seem to modify the association between cruciferous vegetable intake and colorectal adenomas.

Chapter 5 describes the results for NQO1. The *NQO1* 609CT genotype was associated with a higher adenoma risk (OR 1.27, 95% CI 1.00-1.62) compared to the 609CC genotype, whereas the 609TT genotype was not (OR 1.03, 95% CI 0.56-1.88). The higher risk with the *NQO1* 609CT-genotype was seen among smokers (OR 1.96, 95% CI 1.40-2.76), but not among non-smokers (OR 0.91, 95% CI 0.62-1.35; interaction p=0.030). Fruit and vegetable consumption did not protect smokers from adenomas and did not interact with the *NQO1* 609C>T polymorphism or the *NFE2L2* polymorphisms. A higher adenoma risk seen with high fruit and vegetable consumption among *NQO1* -718GG genotypes was absent among -718GA genotypes (p for interaction=0.071). Gene-gene interactions were observed between the *NQO1* 609C>T and *NFE2L2* -686A>G polymorphisms (interaction p=0.056) and between the *NQO1* -718 G>A and *NFE2L2* -650C>A polymorphisms (p for interaction=0.013).

In **chapter 6**, the main findings are summarized and placed in a broader context of methodology and concepts, and their implications are discussed. The focus of the chapter is on the concept of polymorphism functionality, interpreted from and structured into different perspectives: molecular, physiological, epidemiological and societal. These represent a continuum of increasing

integration levels. Emphasis is on the physiological and epidemiological perspective.

It remains uncertain whether the *GSTA1*, *GSTP1* and *NQO1* genes contain regulatory polymorphisms with experimentally shown functional induction differences in relation to fruit and vegetable components (molecular perspective).

It was, however, clearly seen that *GSTA1*, *GSTP1* and *NQO1* contain polymorphisms with consequences for enzyme phenotype. Different botanically defined fruit and vegetable subtypes may be capable of upregulating or downregulating enzyme phenotype. The effect of polymorphisms and fruit and vegetable consumption may be interactive in enzyme phenotype, but this could not clearly be shown. White blood cells are probably not a good surrogate tissue for rectum with respect to GST and NQO1 enzyme phenotype (physiological perspective).

The lower enzyme activity for *GSTP1*/*GSTA1* and *NQO1* that is the result of genetic variation may lead to a slightly increased colorectal adenoma risk in combination with high consumption of certain fruits and vegetables. Methodologically, it cannot be ruled out that specific fruits and vegetables actually confer some unfavourable effects in certain subgroups, but it also cannot be ruled out that some unknown confounding factor or selection bias plays a role (epidemiological perspective).

It is by no means clear that polymorphisms will ever have a legitimate place in (nutritional) prevention strategies. If they should, we must take social, legal and ethical issues into account (societal perspective).

The relation between fruit and vegetable consumption and biotransformation enzymes in cancer prevention is complex. From the example of two important biotransformation enzyme systems, it is not clear what the true integrated effect of total fruit and vegetable consumption, or their subtypes, is on the capacity to detoxify carcinogens in the colorectum. Information contributing to the establishment of dietary advice on a high-risk group level (let alone on an individual level) to prevent colorectal cancer was not delivered. In a larger picture, fruit and vegetable components may affect an array of anti-cancer mechanisms besides biotransformation, may affect organs (and thus cancer sites) differently and may affect disease etiologies differently. The total picture is not clear yet. On a population level, diverse high fruit and vegetable consumption is found to be beneficial for human health. Whether there are individual exceptions to this paradigm remains to be seen.

Samenvatting

In het onderzoek dat in dit boekje beschreven wordt, is een grote rol weggelegd voor het begrip 'genetische variatie'. Onderliggende aanleiding voor het onderzoek is het voorkómen van darmkanker. Hieronder worden eerst de begrippen 'genetische variatie' en 'kanker' kort toegelicht en vervolgens wordt het proefschrift samengevat.

Beide begrippen, genetische variatie en kanker, hebben betrekking op DNA. Het DNA is een code, aanwezig in elke cel, die uitgedrukt wordt in de letters A, G, C en T. Deze aaneenschakeling van letters bevat de informatie voor de aanmaak en het functioneren van allerlei stoffen die tezamen ons lichaam draaiende houden.
Genetische variatie. De DNA code bestaat uit ongeveer 3 miljoen letters. Er zijn per cel twee versies van de DNA code, 1 geërfd van de vader en 1 van de moeder. Aan de basis van elk kenmerk staan dus eigenlijk twee codes die het tot uiting komen ervan bepalen. Die twee stukjes informatie kunnen voor sommige letters verschillen: dat is genetische variatie. Als de stukjes verschillen, dan ben je 'heterozygoot' (bijv. 'AG'). Als de twee stukjes hetzelfde zijn, dan ben je 'homozygoot' (bijv. 'AA'). Twee individuen kunnen ook homozygoot zijn voor een ander stukje (bijv. individu 1 'AA' en individu 2 'GG'). Een heel zichtbaar voorbeeld van genetische variatie is het kenmerk 'oogkleur'. Maar ook bijv. in de aanmaak en het functioneren van een (ontgiftings)eiwit kan genetische variatie een rol spelen. De plek van de variatie kan ook nog een rol spelen. Er is meer en minder functioneel DNA, dus met meer of minder consequenties als er een verandering in is. Grofweg zijn er twee typen functioneel DNA: coderend en regulerend DNA. Coderend DNA bevat de code voor de bouw van eiwitten, welke allerlei belangrijke functies in het lichaam vervullen. Regulerend DNA zorgt er voor dat de eiwitten op het juiste moment in- of juist uitgeschakeld worden.
Kanker. Kanker is een ziekte van het DNA. Elke cel zorgt er normaliter zorgvuldig voor, met de hulp van allerlei ingebouwde beschermingsmechanismen, dat het DNA in goede staat blijft. Dit is erg belangrijk, want als het DNA aangetast wordt, kan het gebeuren dat ook het mechanisme, dat verantwoordelijk is voor de celgroei, wordt ontregeld en dat de natuurlijke rem hierop verloren gaat. De cel gaat dan in het wilde weg groeien en andere cellen, weefsels en organen overwoekeren. Dit is kanker. De cellen van de dikke darm vernieuwen heel vaak en hierdoor kan de groei vaker fout gaan. Daarnaast staan ze ook veel in contact met stoffen van buitenaf. De dikke darm is daarom erg kwetsbaar voor ongezonde voedingspatronen.

Samenvatting van het proefschrift. Achtergrond. Dikke darm kanker is een belangrijke oorzaak van ziekte en sterfte in Nederland en in de wereld. Er wordt gedacht dat consumptie van groenten en fruit beschermt tegen het ontstaan van

dikke darm tumoren. Een mogelijke reden hiervoor is dat het de aanmaak van ontgiftingseiwitten bevordert. Ontgiftingseiwitten, zoals glutation-S-transferases (GSTs) en NAD(P)H:quinon oxidoreductase (NQO1). Deze ontgiftingseiwitten pakken stoffen aan die schadelijk zijn voor het DNA en zorgen er voor dat de hoeveelheid van deze stoffen niet te groot worden. Zo wordt voorkomen dat deze stoffen schade aan DNA aanrichten en daarmee de kans op kanker vergroten.

Eerder onderzoek. In welke mate groenten en fruit beschermend werken is echter onduidelijk. Een verklaring hiervoor zou kunnen zijn dat het erg moeilijk is om de consumptie van groenten en fruit te meten. Een andere mogelijke verklaring is dat er tussen mensen genetische verschillen zijn in de manier waarop de ontgiftingseiwitten reageren op groenten en fruit. Eerdere studies hebben laten zien dat de hoeveelheid en de activiteit van de GSTs hoger wordt na de consumptie van groenten en fruit, met name van koolsoorten en ui-achtigen. In de meeste studies is slechts naar 1 type eiwit en 1 type groente of fruit gekeken. Meestal is niet in de darm gekeken, maar alleen in bloed. Ook is niet vaak naar genetische variatie gekeken. Met betrekking tot NQO1 zijn heel weinig gegevens bekend. Tevens is weinig informatie beschikbaar over het samenspel tussen groenten en fruit en genetische variatie in het ontstaan van dikke darmkanker of darmpoliepen, een mogelijk voorstadium van kanker. Verder is niet bekend of er genetische variatie is specifiek in regulerende regio's van de genen.

Het huidige onderzoek. In de studies die in dit proefschrift beschreven worden, is niet daadwerkelijk naar kanker gekeken. Eén uitkomstmaat was de hoeveelheid en activiteit van de GST en NQO1 ontgiftingseiwitten in het laatste stukje van de dikke darm (eindpunt 1). Er is onderzocht wat de invloed is van groente- en fruitconsumptie en genetische variatie in GSTA1, GSTP1, en NQO1 hierop. Ook is dit bekeken in witte bloedcellen. Een andere uitkomstmaat was de aanwezigheid van darmpoliepen (eindpunt 2), die op zichzelf niet kwaadaardig zijn, maar waarvan wel gedacht wordt dat ze bij veel darmkankers een voorstadium zijn geweest. Er is onderzocht hoe groente- en fruitconsumptie en genetische variatie mogelijk samenspelen bij het ontstaan hiervan. Dit kan meer inzicht geven in bepaalde aspecten rondom het ontstaan van kanker. Ook kan het er toe leiden dat bepaalde groepen mensen geïdentificeerd worden die meer baat hebben bij het eten van groenten en fruit.

Wat betreft de eerste uitkomstmaat, de hoeveelheid en activiteit van de ontgiftingseiwitten, werden de volgende onderzoeksvragen geformuleerd:

1) Zijn er genetische variaties in de regulerende en coderende gebieden van GSTP1, GSTA1 en NQO1, die samenhangen met verschillen in GST- en NQO1-hoeveelheden en -activiteiten?

2) Houdt recente consumptie van groenten en fruit verband met verschillen GST- en NQO1-hoeveelheden en -activiteiten?
3) Kan een hoge groente- en fruitconsumptie de GST en NQO1 hoeveelheid en activiteit veranderen in combinatie met specifieke groepen van genetische variatie?
4) Is de hoeveelheid en activiteit van de GSTs en NQO1 in witte bloedcellen een goede afspiegeling van de hoeveelheid en activiteit van de GST's en NQO1 in de darm?

Om deze vragen te beantwoorden, is informatie over de voeding verzameld en zijn stukjes darmweefsel en bloed onderzocht bij 94 mensen, die de polikliniek bezochten voor een kijkonderzoek van de dikke darm. Bij dezen mensen zijn genetische variaties in verschillende GST's, in NQO1 en in NFE2L2 bepaald en de recente inname van groenten en fruit is gemeten door middel van een voedingsdagboekje. De hoeveelheid en activiteit van de GSTs en NQO1 is gemeten in weefsel van het laatste stukje van de dikke darm, d.w.z. het rectum, en in witte bloedcellen. Wat betreft NQO1 is in de darm ook mRNA gemeten, een soort overgangsvorm tussen DNA en eiwitten.

In **hoofdstuk 2** worden de resultaten beschreven voor de GST's. GST hoeveelheden in het rectum verschilden tussen degenen die recent ui-achtigen, komkommer-achtigen, schermbloem-achtigen en citrus-fruit hadden gegeten en degenen die dat niet hadden gedaan. De GST activiteit in het rectum werd echter niet duidelijk beïnvloed door groente- en fruitconsumptie. De activiteit werd voornamelijk bepaald door een genetische variatie in GSTP1 (313A>G): vergeleken met de 313AA varianten, hadden de 313AG en 313GG varianten respectievelijk een 36 en 67 nmol/min.mg eiwit lagere GST activiteit. De correlatie tussen de activiteit in de darm en in witte bloedcellen was laag (r=0,4) en de verscheidene genetische en voedingsfactoren leken een andere betekenis te hebben voor de twee weefsels.

Hoofdstuk 4 beschrijft de resultaten voor NQO1. De inname van groenten en fruit ging niet gepaard met meer mRNA of een grotere eiwitactiviteit, maar eerder met minder mRNA en een lagere activiteit. De NQO1 609CC varianten hadden een hogere rectale NQO1 activiteit vergeleken met de 609CT varianten (er waren geen 609TT varianten). Er was een correlatie tussen mRNA en activiteit voor de 609CC varianten (r=0,5), maar niet voor de 609CT varianten (r=0,14). De NFE2L2 -684A-variant hing samen met een hogere hoeveelheid NQO1 mRNA. De andere varianten beïnvloedden mRNA of activiteit niet. De NQO1 activiteit in rectum correleerder niet met de activiteit in witte bloedcellen.

Met betrekking tot de tweede uitkomstmaat, de aanwezigheid van darmpoliepen, werden de volgende onderzoeksvragen geformuleerd:

1) Zijn er genetische variaties in de regulerende en coderende gebieden van GSTP1, GSTA1 en NQO1, die samenhangen met de aan- of afwezigheid van darmpoliepen?
2) Hangt consumptie van groenten en fruit samen met de aan- of afwezigheid van darmpoliepen?
3) Hangt groente- en fruitconsumptie samen met een verschil in de aan- of afwezigheid van darmpoliepen tussen verschillende genotypes? Met andere woorden, is er een samenspel tussen consumptie en genetische factoren?

Dit is als volgt onderzocht. Onder 1477 mensen die de polikliniek bezochten voor een kijkonderzoek van de dikke darm is een patiënt-controle vergelijking gemaakt. Genetische variaties in verschillende GST's, in NQO1 en in NFE2L2 zijn bepaald en de dagelijkse inname van groenten en fruit is gemeten door middel van een voedselfrequentie vragenlijst.

In **hoofdstuk 3** worden de resultaten van de GSTs beschreven. Een hoge inname van koolsoorten hing licht samen met een grotere kans op darmpoliepen. Kijkend naar GSTP1, dan was dit alleen zo voor mensen met het GSTP1 313GG genotype, dat gepaard gaat met een lagere GST activiteit. Dit was tegengesteld aan onze hypothese. Het samenspel was duidelijker te zien bij mannen, bij hogere leeftijd en bij hogere vleesinname. De GSTA1 variatie zou ook een modificerende rol kunnen spelen: de kans op darmpoliepen was hoger bij hogere kool-inname voor mensen met het -69TT genotype, dat gepaard gaat met een lagere hoeveelheid eiwit. Dit leek sterker te zijn bij een lagere leeftijd en hogere vleesinname. Voor mensen met varianten van beide genotypes was het samenspel statistisch significant. Voor de GSTM1 en GSTT1 varianten kon er geen dergelijk samenspel ontdekt worden.

Hoofdstuk 5 beschrijft de resultaten voor NQO1. Het NQO1 609CT genotype hing samen met een groter darmpoliep risico vergeleken met het 609CC genotype, maar het 609TT genotype had geen groter risico. Dit grotere risico was zichtbaar bij rokers, maar niet bij niet-rokers. Groente- en fruitconsumptie beschermde rokers niet. Er was geen samenspel met de NQO1 609C>T en NFE2L2 variaties. Het was wel opvallend dat het hogere polieprisico dat werd gezien bij hogere consumptie van groenten en fruit voor mensen met het NQO1 -718GG genotype, niet werd gezien bij mensen met het -718GA genotype. Er leek een samenspel te zijn tussen de NQO1 609C>T en NFE2L2 -686 varianten en tussen de NQO1 -718 en NFE2L2-650 varianten).

In **hoofdstuk 6** worden de hoofdbevindingen nog eens op een rijtje gezet, in bredere context geplaatst en worden de implicaties besproken. Uitgangspunt hierbij is het concept 'functionaliteit'. Dit concept wordt in verschillende

perspectieven geplaatst: moleculair, fysiologisch, epidemiologisch en maatschappelijk. Deze perspectieven weerspiegelen een continuüm van niveaus die steeds meer geïntegreerd zijn. De nadruk is op het fysiologische en epidemiologische perspectief.

Het blijft onduidelijk of de onderzochte genen variaties bevatten in het regulerende DNA met experimenteel aangetoonde verschillen in hun reactie op groente- en fruitcomponenten (moleculair perspectief).

De GSTs en NQO1 bevatten wel duidelijk variaties die gevolgen hebben voor de hoeveelheid en activiteit van de eiwitten. Verschillende typen groenten en fruit zouden de ontgiftingseiwitten kunnen bevorderen of juist tegengaan. Wellicht is er een samenspel tussen groenten en fruit en genetische variaties, maar dit kon niet duidelijk aangetoond worden. Witte bloedcellen zijn geen goed surrogaatweefsel voor de dikke darm wat betreft GSTs en NQO1 (fysiologisch perspectief).

De lagere eiwitactiviteit voor GSTP1/GSTA1 en NQO1, het resultaat van een genetische variatie, zou in combinatie met hogere groente- en fruitinname kunnen leiden tot een groter risico op darmpoliepen. Methodologisch kan het niet uitgesloten worden dat bepaalde groente- en fruitsoorten ongunstige effecten opleveren in bepaalde subgroepen, maar het kan ook niet uitgesloten worden dat een onbekende verstorende variabele of selectie 'bias' een rol speelt (epidemiologisch perspectief).

Het is nog niet zo vanzelfsprekend dat genetische variaties ooit terecht een plaats krijgen in (op voeding gerichte) preventie strategieën. Hoe dan ook moeten sociale, wettelijke en ethische zaken ook in beschouwing genomen worden.

De relatie tussen groente- en fruitconsumptie en ontgiftingseiwitten in kanker preventie is complex. Uit het voorbeeld van de twee belangrijke onderzochte ontgiftingssystemen komt niet duidelijk naar voren wat het werkelijke, geïntegreerde effect is van groente- en fruitconsumptie op het vermogen van de darm om schadelijke stoffen te ontgiften. De studies hebben geen informatie opgeleverd om voedingsadvies te kunnen geven aan bepaalde genetische hoog-risico groepen (laat staan op individueel niveau) om dikke darm kanker te voorkomen. In het grotere geheel beschouwd, kunnen groente- en fruitcomponenten een scala aan kankerbeschermende mechanismen beïnvloeden, naast ontgifting, kunnen ze verschillende organen anders beïnvloeden en kunnen ze verschillende ziekten anders beïnvloeden. Het totale plaatje is nog onduideljk. Op populatie-niveau wordt gevarieerde consumptie van groenten en fruit als gunstig gezien voor de gezondheid van de mens. Of hier op individueel niveau uitzonderingen op zijn, valt nog te bezien.

Dankwoord

Dit proefschrift kwam tot stand door de bijdrage van velen.
Iedereen hartelijk dank daarvoor!
Hieronder noem ik, met een greep uit vele mogelijke woorden:

DANK

Co-promotoren:
Ellen Kampman: voor alle kennis en kunde
Jac Aarts: voor grondige inzet en openheid voor vakoverstijgende zaken
Promotor:
Frans Kok: voor inzicht en structuur

Projectdeel Toxicologie, Wageningen Universiteit:
Ivonne Rietjens: voor de welkome kritische opmerkingen
Anne-Marie Boerboom: voor het werk in het moleculaire deel van het project
Liesbeth Op den Camp: voor het harde werk in het lab

Humane Voeding, Wageningen Universiteit:
Marleen Visker: voor de belangrijke bijdragen aan het project, van sequencing tot kritisch co-auteurschap
Elgin Lichtenauer: voor de hulp bij de NQO1 genotypering
Lucy Okma: voor de ondersteuning bij de benodigde labmaterialen
Afdeling Diëtetiek: voor de voedingsgegevens, en met name Petra Vissink: voor de flexibiliteit en het onderbreken van vrije dagen
Dione Bouchaut: voor de hulp bij de figuren

GF-studie:
De GF-studie deelnemers: voor alle inzet onder lastige omstandigheden
UMC St. Radboud Universiteit te Nijmgen:
Fokko Nagengast: voor de mogelijkheid om de GF-studie te starten en uit te voeren
Wilbert Peters, Hennie Roelofs, René te Morsche en Annie van Schaik:
voor de ondersteuning op het lab MDL; Wilbert: voor de GST-kennis
Marloes Berkhout: voor de gezelligheid op de Nijmeegse werkplek
Endoscopie: voor de flexibele houding tijdens het werk; speciale dank voor de bloedafnames
Canisius-Wilhelmina Ziekenhuis te Nijmegen:
Adriaan Tan: voor de mogelijkheid om de GF-studie uit te voeren en verdere hulp
Endoscopie: voor de flexibele houding tijdens het werk

POLIEP-studie:
De POLIEP-studie deelnemers: voor alle inzet
De ziekenhuis-medewerkers: voor het mogelijk maken van de POLIEP-studie
Het POLIEP-team van Humane Voeding: Petra Wark, Maureen van den Donk, Edine Tiemersma, Brenda Diergaarde, Maria van Vugt: voor de samenwerking
Jan Harryvan en Annelies Bunschoten: voor alles rondom DNA en genotyperingen

Eva van Soest, Cordny Nederkoorn, Wijnand Laan en Bram-Sieben Rosema: voor het meedenken en het praktische werk tijdens de MSc-projecten

Mijn mede-aio's: voor luisterende oren en gezellige momenten
Speciale dank voor de PhD-tour groep van 2005: super-reis

Mijn achtereenvolgende kamergenoten Siti Muslimatun, Mark Boekschoten en Cora Busstra: voor jullie gezelschap
Speciale dank aan Cora: voor het aanhoren van frustraties en kolf-gezoem, voor de lay-out aanwijzingen welke de realisatie van dit proefschrift erg geholpen hebben

Annemiek Nelis, Franck Meijboom, Frans Brom: voor de uitbreiding van mijn inzichten op gebied van ethiek en sociale wetenschappen

Mijn RIVM collega's: voor de interesse en sfeer

Speciaal nog eens: mijn paranimfen Maureen en Marleen; Maureen: voor het verblijden van de tijd in Wageningen, Marleen: voor alle positieve woorden.
Beide dank dat jullie naast mij willen staan

Vrienden en familie: voor alle steun, op velerlei manieren.
Speciale dank aan:
Mijn vriendinnetjes: voor alle zorgen en gezelligheid
Mimi en Camille: voor jullie belangstelling en steun. Camille: voor de wetenschap dat de kaakjes met Jens fantastisch zouden zijn geweest
Mijn ouders: voor het besef dat jullie altijd bereid zijn te helpen en voor alle hulp die jullie al geboden hebben
Jens: voor je enorme enthousiasme en vrolijkheid, voor je knuffeltjes en kusjes
Milo: voor zó veel, voor de blijdschap om thuis te zijn

Mariken

Curriculum Vitae

ABOUT THE AUTHOR

Mariken Joan Tijhuis was born on 14th April 1976 in Enschede, The Netherlands. She completed secondary school (St. Willibrord Gymnasium, Deurne) in 1994. In 1995 she started her studies of Health Sciences at the University of Maastricht, specialising in Nutrition and in Epidemiology. Her MSc research and thesis were on 'nutrition, homocysteine and heart failure'. In 2001 she started the PhD-project of which this thesis is the final result. It comprised the conduct of observational studies, the writing of scientific publications and the attendance of several courses and conferences, see below. Mariken is currently employed at the Center for Nutrition and Health at the National Institute for Public Health and the Environment in Bilthoven.

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TRAINING

Discipline-specific activities

- Masterclass Nutrigenomics, WUR/VLAG/OSPT/HNE, 2001
- Molecular Epidemiology, IARC, 2001
- Geriatric Nutrition, VLAG, 2004
- Searching Genes of Complex Disorders, ESP, 2004
- Bioinformatics in Medicine, ESP, 2004
- History of Epidemiological Ideas, ESP, 2005
- Annual Epidemiology Symposium (WEON) 2001, 2002, 2005
- Nutrition and Cancer Conferences, Lyon 2001, Washington DC 2005
- Meetings of Learned Societies on Nutrition, Cancer, SNPs, 2001-2006

General courses

- VLAG PhD Week, 2001
- Organising and supervising MSc thesis work, WUR, 2003
- Regression Analysis, ESP, 2005
- The Future Genomics society, VLAG, MGS, Biosystems genomics, Society & Genomics and WCFS-CMSB, 2005
- International Seminar: Genetically Tailor-made Diets: future prospects and ethical challenges, Bio-ethics Institute Utrecht University, 2005

Optional courses and activities

- Preparation of Research Proposal
- Journal Club, 2001-2005
- PhD Study Tour UK & Republic of Ireland, 2005

The project described in this thesis was financially supported by the Netherlands Organisation for Health Research and Development (ZonMW), grant number: 21000054, and the Dutch Digestive Diseases Foundation (MLDS), grant number WS 00-31

The printing of this thesis was financially supported by Wageningen University

Printing: Ponsen en Looijen BV, Wageningen

Cover: Prithivi & Akasha